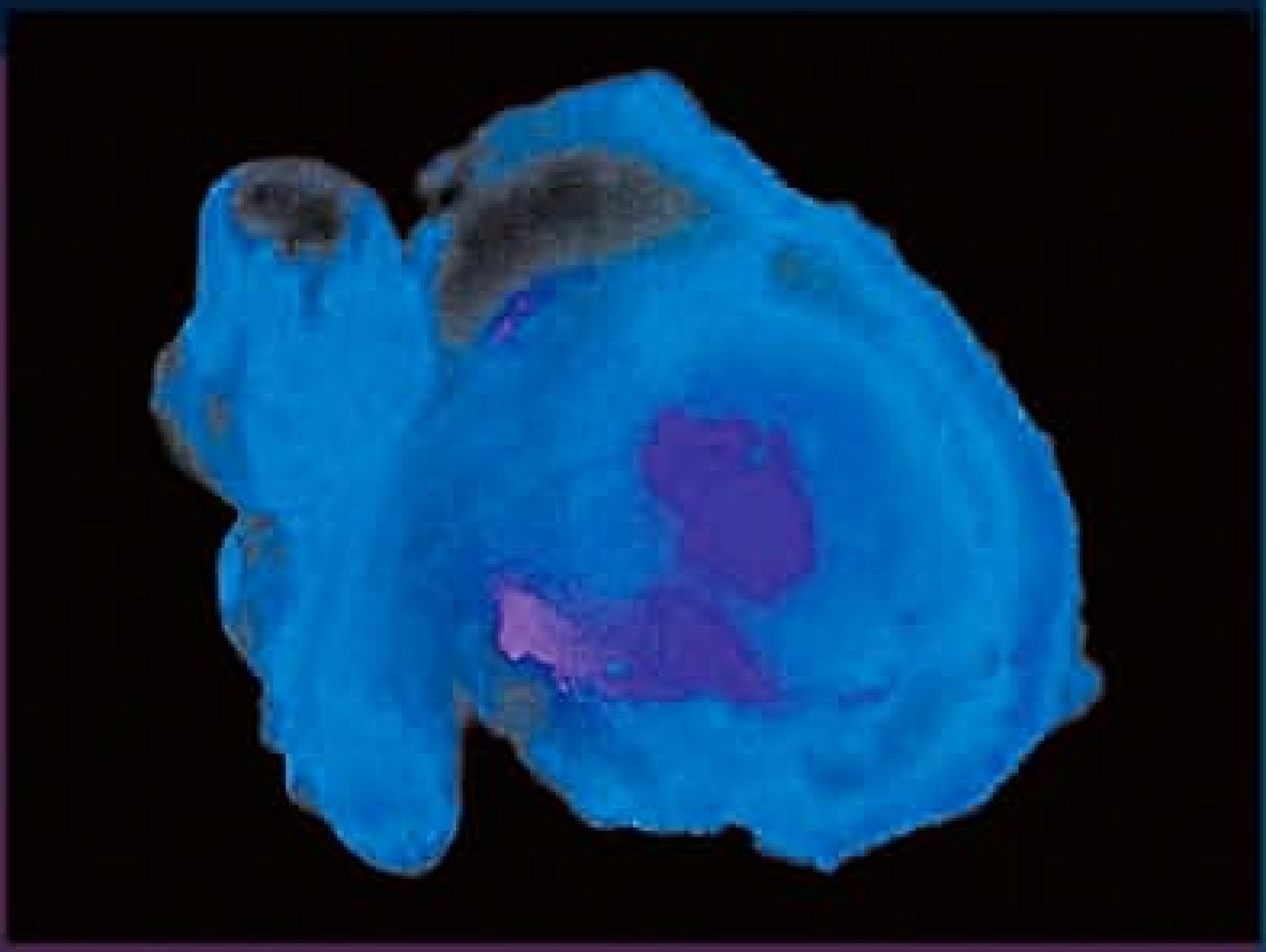
Essentials of
STEM CELL BIOLOGY

2nd Edition



Edited by ROBERT LANZA

John Gearhart • Brigid Hogan • Douglas Melton • Roger Pedersen
E. Donnall Thomas • James Thomson • Sir Ian Wilmut

VISIT…



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Contributors

Numbers in parentheses indicate the chapter number of the authors' contribution.

Russell C. Addis (42) John Hopkins University, School of Medicine, Baltimore, MD

Michal Amit (40) Department of Obstetrics and Gynecology, Rambam Medical Center, and The Bruce Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel

Peter W. Andrews (11, 47) Department Biomedical Science, University of Sheffield, Western Bank, Sheffield, UK

Piero Anversa (59) Departments of Anesthesia and Medicine, and Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Anthony Atala (16) Wake Forest Institute for Regenerative Medicine, Wake University School of Medicine, Winston-Salem, NC

Joyce Axelman (42) John Hopkins University, School of Medicine, Baltimore, MD

Anne G. Bang (38) Novocell, Inc., 3500 General Atomics Ct, San Diego, CA

Yann Barrandon (61) Laboratory of Stem Cell Dynamics, School of Life Sciences, Swiss Federal Institute of Technology Lausanne and Department of Experimental Surgery, Lausanne University Hospital 1015, Lausanne, Switzerland

Steven R. Bauer (68) Laboratory of Stem Cell Biology, Division of Cellular and Gene Therapies, Office of Cellular, Tissue and Gene Therapies, Center for Biologics Evaluation and Research, US Food and Drug Administration, Rockville, MD

Daniel Becker (55) Department of Neurology, Johns Hopkins School of Medicine and Kennedy Krieger Institute, 707 North Broadway, Suite 518, Baltimore, MD

Nissim Benvenisty (45) Department of Genetics, Silberman Institute of Life Sciences, The Hebrew University, 91904 Jerusalem, Israel

Paolo Bianco (64) Dipartimento di Medicina Sperimentale, Sapienza, Universita di Roma, Rome, Italy; Parco Scientifico Biomedico San Raffaele, Rome, Italy

Helen M. Blau (30) Baxter Laboratory in Genetic Pharmacology, Dept of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

Susan Bonner-Weir (57) Section on Islet Transplantation and Cell Biology, Joslin Diabetes Center, Harvard Medical School, Boston, MA

Mairi Brittan (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK

Hal E. Broxmeyer (17) Walther Oncology Center and Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN

Scott Bultman (10) Department of Genetics, University of North Carolina at Chapel Hill, NC

Arnold I. Caplan (29) Skeletal Research Center, Case Western Reserve University, Cleveland, OH

Melissa K. Carpenter (38, 44) Carpenter Group, 10330 Wateridge Circle #290, San Diego, CA

Fatima Cavaleri (6) Max Planck Institute for Molecular Biomedicine, Muenster, Germany

Connie Cepko (21) Department of Genetics, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA

Howard Y. Chang (51) Department of Dermatology and Program in Epithelial Biology, Stanford University School of Medicine, Stanford, CA

Xin Chen (51) Department of Biopharmaceutical Sciences, University of California, San Francisco, CA, USA

Tao Cheng (9) Massachusetts General Hospital, Harvard Medical School, Boston, MA

Susana M. Chuva de Sousa Lopes (13, 14) Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands

Gregory O. Clark (42) Division of Endocrinology, John Hopkins University, School of Medicine, Baltimore, MD

Michael F. Clarke (53) Stanford Institute for Stem Cell and Regenerative Medicine; Department of Medicine, Division of Oncology, Stanford University School of Medicine, Stanford, CA

Giulio Cossu (60) Stem Cell Research Institute, Dibit, H.S. Raffaele, Milan, Italy

Annelies Crabbe (28) Interdepartementeel Stamcelinstituut, Katholieke Universiteit Leuven, Belgium

George Q. Daley (24) Children's Hospital Boston, MA

Ayelet Dar (27) Stem Cell Center, Bruce, Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

Brian R. Davis (65) Centre for Stem Cell Research, Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center, Houston, TX

Natalie C. Direkze (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK Histopathology Unit, London Research Institute, Cancer Research UK, London, UK

Yuval Dor (35) Department of Cellular Biochemistry and Human Genetics, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Jonathan S. Draper (47) Department Biomedical Science, University of Sheffield, Western Bank, Sheffield, UK

Gregory R. Dressler (33) Department of Pathology, University of Michigan, Ann Arbor, MI

Martin Evans (39) Cardiff School of Biosciences, Cardiff University, Cardiff, UK

Margaret A. Farley (67) Yale University Divinity School, New Haven, CT

Donna Fekete (21) Department of Biological Sciences, Purdue University, West Lafayette, IN

Qiang Feng (25) Stem Cell and Regenerative Medicine International, 381 Plantation Street, Worcester, MA

Loren J. Field (56) The Riley Heart Research Center, Herman B Wells Center for Pediatric Research; the Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN

Donald W. Fink (68) Cell Therapy Branch, Division of Cellular and Gene Therapies, Office of Cellular, Tissue and Gene Therapies, Center for Biologics Evaluation and Research, US Food and Drug Administration, Rockville, MD

K. Rose Finley (52) Howard Hughes Medical Institute, Children's Hospital, Boston, MA

Elaine Fuchs (22) Howard Hughes Medical Institute, Laboratory of Mammalian Cell Biology and Development, The Rockefeller University, New York, NY

Margaret T. Fuller (7) Stanford University School of Medicine, Department of Developmental Biology, Stanford, CA

Richard L. Gardner (1) University of Oxford, Dept of Zoology, Oxford, UK

John D. Gearhart (42) Institute for Regenerative Medicine, University of Pennsylvania, Philadelphia, PA

Pamela Gehron Robey (64) Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, Bethesda, MD

Sharon Gerecht-Nir (27) Stem Cell Center, Bruce Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel

Penney M. Gilbert (30) Baxter Laboratory in Genetic Pharmacology, Dept of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

Victor M. Goldberg (62) Department of Orthopaedics, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, OH

Rodolfo Gonzalez (54) Program in Stem Cell Biology (Developmental & Regeneration Cell Biology), The Burnham Institue, La Jolla, CA

Elizabeth Gould (20) Department of Psychology, Princeton University, Princeton, NJ

Trevor A. Graham (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK

Ronald M. Green (66) Ethics Institute, Dartmouth College, Hanover, NH

Markus Grompe (34) Papé Family Pediatric Research Institute, Oregon Stem Cell Center, Oregon Health & Science University, Portland, OR

Dirk Hockemeyer (4) The Whitehead Institute, 9 Cambridge Center, Cambridge, MA

Marko E. Horb (12) Centre for Regenerative Medicine, Department of Biology & Biochemistry, University of Bath, Bath, UK

Jerry I. Huang (62) University Hospitals Research Institute, Cleveland, OH

Adam Humphries (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK

Joseph Itskovitz-Eldor (27, 40) Department of Obstetrics and Gynecology, Rambam Medical Center, and The Bruce Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel

Rudolf Jaenisch (4) The Whitehead Institute, Cambridge, MA; Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

Penny Johnson (11) Department Biomedical Science, University of Sheffield, Western Bank, Sheffield, UK

D. Leanne Jones (7) Stanford University School of Medicine, Department of Developmental Biology, Stanford, CA

Jan Kajstura (59) Departments of Anesthesia and Medicine, and Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Gerard Karsenty (26) Baylor College of Medicine, Houston, TX

Pritinder Kaur (58) The University of Melbourne, Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia

Kathleen C. Kent (42) Johns Hopkins University, School of Medicine, Baltimore, MD

Candace L. Kerr (42) Department of Gynecology and Obstetrics, John Hopkins University, School of Medicine, Baltimore, MD

Ali Khademhosseini (63) Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA

Chris Kintner (18) The Salk Institute for Biological Studies, San Diego, CA

Irina Klimanskaya (41) Advanced Cell Technology, 381 Plantation Street, Worcester, MA

Naoko Koyano-Nakagawa (18) Stem Cell Institute, Department of Neuroscience, University of Minnesota, Minneapolis MN, USA

Jennifer N. Kraszewski (42) Johns Hopkins University, School of Medicine, Baltimore, MD

Tilo Kunath (15) Mount Sinai Hospital, Toronto, Ontario, Canada

Robert Langer (63) Langer Lab, Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA

Robert Lanza (25) Advanced Cell Technology, and Stem Cell and Regenerative Medicine International, 381 Plantation Street, Worcester, MA

Annarosa Leri (59) Departments of Anesthesia and Medicine, and Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Shulamit Levenberg (63) Faculty of Biomedical Engineering Techion, Haifa, Israel

S. Robert Levine (69) Research Portfolio Committee, Juvenile Diabetes Research Foundation

Olle Lindvall (5) Laboratory of Neurogenesis and Cell Therapy, Section of Restorative Neurology, Wallenberg Neuroscience Center, University Hospital, Lund, Sweden; Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund, Sweden

John W. Littlefield (42) Johns Hopkins University, School of Medicine, Baltimore, MD

Shi-Jiang Lu (25) Advanced Cell Technology, and Stem Cell and Regenerative Medicine International, 381 Plantation Street, Worcester, MA

Terry Magnuson (10) Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC

Yoav Mayshar (45) Department of Genetics, Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, Israel

John W. McDonald (55) Department of Neurology, Johns Hopkins School of Medicine and Kennedy Krieger Institute, 707 North Broadway, Suite 518, Baltimore, MD

Stuart A. C. McDonald (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK; Histopathology Unit, London Research Institute, Cancer Research UK, London, UK

Anne McLaren (14) The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK. Tragically died on 7th of July 2007

Jill McMahon (41) Harvard University, 16 Divinity Ave, Cambridge, MA

Douglas A. Melton (35) Department of Molecular and Cellular Biology and Howard Hughes Medical Institute, Harvard University, Cambridge, MA

Christian Mirescu (20) Department of Psychology, Princeton University, Princeton, NJ

Nathan Montgomery (10) Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC

Malcolm A. S. Moore (23) Developmental Hematology, Memorial Sloan-Kettering Cancer Center, New York, NY

Mary Tyler Moore (69) International Chairwoman, Juvenile Diabetes Research Foundation

Christine L. Mummery (13) Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands

Andras Nagy (48) Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, Canada

Satomi Nishikawa (32) Stem Cell Research Group, Riken Center for Developmental Biology, Kobe, Japan

Shin-Ichi Nishikawa (32) Stem Cell Research Group, Riken Center for Developmental Biology, Kobe, Japan

Hitoshi Niwa (8) Lab for Pluripotent Cell Studies, RIKEN Ctr for Developmental Biology, Chu-o-ku, Kobe C, Japan

Jennifer S. Park (25) Advanced Cell Technology, 381 Plantation Street, Worcester, MA

Ethan S. Patterson (42) Johns Hopkins University, School of Medicine, Baltimore, MD

Alice Pébay (43) Centre for Neuroscience and Department of Pharmacology, The University of Melbourne, Parkville, Victoria, Australia

Martin F. Pera (43) Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, CA

Christopher S. Potten (3) EpiStem Limited, Incubator Building, Manchester, UK

Bhawana Poudel (31) Heart Science Centre, NHLI Division, Imperial College London, UK

Sean L. Preston (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK; Histopathology Unit, London Research Institute, Cancer Research UK, London, UK

Nicole L. Prokopishyn (65) Calgary Laboratory Services, Foothills Medical Centre, Calgary, Alberta, Canada

Emily K. Pugach (52) Howard Hughes Medical Institute, Children's Hospital, Boston, MA

Jean Pyo Lee (54) Program in Stem Cell Biology (Developmental & Regeneration Cell Biology) The Burnham Institue, La Jolla, CA

Ariane Rochat (61) Laboratory of Stem Cell Dynamics, School of Life Sciences, Swiss Federal Institute of Technology Lausanne and Department of Experimental Surgery, Lausanne University Hospital 1015, Lausanne, Switzerland

Nadia Rosenthal (31) Heart Science Centre, NHLI Division, Imperial College London, UK; Mouse Biology Unit European Molecular Biology Laboratory, Monterotondo (Rome) Italy; Australian Regenerative Medicine Institute, Monash University, Melbourne, Australia

Janet Rossant (2, 15) Mount Sinai Hospital, Toronto, Ontario, Canada

Michael Rothenberg (53) Stanford Institute for Stem Cell and Regenerative Medicine; Department of Medicine, Stanford University School of Medicine; Division of Gastroenterology and Hepatology, Stanford University School of Medicine, Stanford, CA

Michael Rubart (56) The Riley Heart Research Center, Herman B Wells Center for Pediatric Research, Indianapolis, IN

Alessandra Sacco (30) Baxter Laboratory in Genetic Pharmacology, Dept of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

Maurilio Sampaolesi (60) Stem Cell Research Institute, Dibit, H.S. Raffaele, Milan, Italy

Maria Paola Santini (31) Heart Science Centre, NHLI Division, Imperial College London, UK

David T. Scadden (9) Massachusetts General Hospital, Harvard Medical School, Boston, MA

Hans Schöler (6) Max Planck Institute for Molecular Biomedicine, Muenster, Germany

Tom Schulz (44) Novocell, Inc., 111 Riverbend Rd, Athens, GA

Michael J. Shamblott (42) Institute for Cell Engineering, Johns Hopkins University, School of Medicine, Baltimore, MD

William B. Slayton (50) University of Florida College of Medicine, Pediatric Hematology/Oncology, Gainesville, FL

Evan Y. Snyder (54) Program in Stem Cell Biology (Developmental & Regeneration Cell Biology) The Burnham Institue, La Jolla, CA

Frank Soldner (4) The Whitehead Institute, 9 Cambridge Center, Cambridge, MA

Gerald J. Spangrude (50) University of Utah, Division of Hematology, Salt Lake City, UT

Lorenz Studer (19) Laboratory of Stem Cell & Tumor Biology, Neurosurgery and Developmental Biology, Memorial Sloan Kettering Cancer Center, New York, NY

M. Azim Surani (49) Wellcome Trust Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK

James A. Thomson (37, 46) Morgridge Institute for Research in Madison, Wisconsin; the University of Wisconsin School of Medicine and Public Health; Molecular, Cellular, and Developmental Biology (MCDB) Department at the University of California, Santa Barbara

David Tosh (12) Centre for Regenerative Medicine, Department of Biology & Biochemistry, University of Bath, Bath, UK

Tudorita Tumbar (22) Howard Hughes Medical Institute, Laboratory of Mammalian Cell Biology and Development, The Rockefeller University, New York, NY

Edward Upjohn (58) The University of Melbourne, Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia

George Varigos (58) The University of Melbourne, Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia

Catherine M. Verfaillie (28) Interdepartementeel Stamcelinstituut, Katholieke Universiteit Leuven, Belgium

Gordon C. Weir (57) Section on Islet Transplantation and Cell Biology, Joslin Diabetes Center, Harvard Medical School, Boston, MA

J. W. Wilson (3) EpiStem Limited, Incubator Building, Manchester, UK

Nicholas A. Wright (36) Centre for Gastroenterology, Institute of Cell and Molecular Sciences, Barts and the London School of Medicine and Dentistry, London, UK; Histopathology Unit, London Research Institute, Cancer Research UK, London, UK

Jun K. Yamashita (32) Laboratory of Stem Cell Differentiation, Institute for Frontier Medical Sciences, Kyoto University; Center for iPS Cell Research and Application, Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan

Holly Young (44) Novocell, Inc., 3500 General Atomics Ct, San Diego, CA

Junying Yu (37) University of Wisconsin School of Medicine and Public Health, WI

Leonard I. Zon (52) Howard Hughes Medical Institute, Children's Hospital, Boston, MA

Thomas P. Zwaka (46) Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX

Preface to the Second Edition

The second edition of *Essentials of Stem Cell Biology* incorporates the latest advances in the field of stem cells, with new chapters on clinical translation, cancer stem cells, and direct reprogramming—including chapters by the scientists whose groundbreaking research ushered in the era of induced pluripotent stem (iPS) cells. While the second edition offers a comprehensive—and much needed—update of the rapid progress that has been achieved in the field in the last half decade, we have retained those facts and subject matter which, while not new, is pertinent to the understanding of this exciting area of biology.

Like the original volume, the second edition of *Essentials of Stem Cell Biology* is presented in an accessible format, suitable for students and general readers interested in following the latest advances in stem cells. The organization of the book remains largely unchanged, combining the prerequisites for a general understanding of embryonic, fetal, and adult stem cells; the tools, methods, and experimental protocols needed to study and characterize stem cells and progenitor populations; as well as a presentation by the world's leading scientists of what is currently known about each specific organ system.

No topic in the field of stem cells is left uncovered, including basic biology/mechanisms, early development, ectoderm, mesoderm, endoderm, methods (such as detailed descriptions of how to generate both iPS and embryonic stem cells), application of stem cells to specific human diseases, regulation and ethics, and a patient perspective by Mary Tyler Moore. The second edition also includes a Foreword by 2007 Nobel laureate Sir Martin Evans (who is credited with discovering embryonic stem cells). The result is a comprehensive reference that we believe will be useful to students and experts alike, and that represents the combined effort of eight editors and more than 200 scholars and scientists whose pioneering work has defined our understanding of stem cells.

Robert Lanza, M.D.
Boston, Massachusetts

Foreword

It is with great pleasure that I pen this foreword to the second edition of the *Essentials of Stem Cell Biology*. The field of stem cell biology is moving extremely rapidly as the concept and potential practical applications have entered the mainstream. Despite this worldwide intensity and diversity of endeavor, there remain a smaller number of definable leaders in the field, and this volume brings most of them together.

Although the concept of stem and progenitor cells has been known for a long time, it was the progress towards embryonic stem cells which lit the field. Mouse embryonic stem (ES) cells originally came from work aimed at understanding the control and progress of embryonic differentiation, but their *in vitro* differentiation, despite being magnificent, was overshadowed experimentally by their use as a vector to the germline, and hence as a vehicle for experimental mammalian genetics. This now has led to studies of targeted mutation in up to one third of gene loci, and an ongoing international program to provide mutation in every locus of the mouse. These studies greatly illuminate our understanding of human genetics.

Jamie Thomson, reporting the advent of the equivalent human embryonic stem cells, very clearly signaled that their utility would be neither in genetic studies (impractical and unethical in man), nor in fundamental studies of embryonic development (already catered for by mouse ES cells), but, by providing a universal source of a diversity of tissue-specific precursors, as a resource for tissue repair and regenerative medicine.

Progress towards the understanding of pluripotentiality and the control of cellular differentiation, that is basic fundamental developmental biology at the cell and molecular level, now stands as a gateway to major future clinical applications. This volume provides a timely, up-to-date state-of-the-art reference.

The ideas behind regenerative medicine, powered by the products of embryonic stem cells, reinvigorated study of committed stem and precursor cells within the adult body. The use of such stem cells in regenerative medicine already has a long history, for example in bone marrow transplantation and skin grafting. In both of these examples not only gross tissue transplantation, but also purified or cultured stem cells may be used. They have been extensively applied in clinical treatment, and have most clearly demonstrated the problems which arise with histoincompatibility. Ideally, in most cases, a patient is better treated with his own—autologous—cells than with partially matching allogeneic cells. An ideal future would be isolation, manipulation, or generation of suitable committed stem or precursor cell populations from the patient for the patient. The amazing advances of induced pluripotential stem cells point to the possibilities of patient-specific *ad hominem* treatment. This personalized medicine would be an ideal scenario, but as yet the costs of the technologies may not allow it to be a commercial way forward. The timelines are, however, likely to be long before the full promise of these technologies is realized, and there is every possibility that such hurdles will be circumvented. Quite properly, much of this book concentrates on the fundamental developmental and cell biology from which the solid applications will arise.

This is a knowledge-based field in which we have come a long way, but are still relatively ignorant. We know many of the major principles of cell differentiation, but as yet need to understand more in detail, more about developmental niches, more about the details of cell–cell and cell growth-factor interaction, and more about the epigenetic programming which maintains the stability of the differentiated state.

Professor Sir Martin Evans



Sir Martin Evans, PhD, FRS
Nobel Prize for Medicine 2007
Sir Martin is credited with discovering embryonic stem cells, and is considered one of the chief architects of the field of stem cell research. His ground-breaking discoveries have enabled gene targeting in mice, a technology that has revolutionized genetics and developmental biology, and have been applied in virtually all areas of biomedicine—from basic research to the development of new medical therapies. Among other things, his research inspired the effort of Ian Wilmut and his team to create Dolly the cloned sheep, and Jamie Thomson's efforts to isolate embryonic stem cells from human embryos, another of the great medical milestones in the field of stem cell research. Professor Evans was knighted in 2004 by Queen Elizabeth for his services to medical science. He studied at Cambridge University and University College London before leaving to become director of bioscience at Cardiff University.

Why Stem Cell Research

Medical research is endlessly exciting, by its very nature continuously uncovering new facts and principles that build upon existing knowledge to modify the way we think about biological processes. In the history of science, certain discoveries have indeed transformed our thinking and created opportunities for major advancement, and so it is with the discovery and isolation of pluripotential stem cells. Although appearing only briefly in mammalian development, they are a source of an organism's complete array of cell types at every stage of development, from embryogenesis through senescence, in health and in disease.

Scientists recognizing the remarkable opportunities pluripotential stem cells provide have, in a less than a decade, progressed from being able to isolate pluripotential stem cells from early embryos and grow the cells in the laboratory (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000), to being able to generate them by reprogramming somatic cells using viral insertion of key transcription factors (Okita *et al.*, 2007; Takahashi *et al.*, 2007). These advances now make it possible, in principle, to use stem cells for cell therapy—to identify new molecular targets for disease treatment, to contain oncogenesis, to reconstruct or replace diseased tissues—and for gene therapy. New opportunities for expanding effective hematopoietic and other adult stem cell therapies appear in the literature almost daily, and increasing numbers of scientists, clinicians, and patient advocates are becoming excited about an impending revolution in non-hematopoietic cell-based medicine.

Embryonic stem (ES) cells will remain the gold standard for pluripotentiality research, but induced pluripotential stem (iPS) cells hold the promise of making personalized medicine a reality. By using them we can analyze the heterogeneity of complex human diseases, including the diverse causes of cell degeneration and cell death—information certain to help us develop new drugs. IPs cells will also help us understand adverse responses to new drugs by those small cohorts within larger patient populations who can stall or collapse otherwise successful clinical trials. Central to these studies will be the need to precisely manipulate cell fate and commitment decisions to create the tissues that are needed, but doing so will require much more information about the cocktails of transcription factors necessary to regulate cell differentiation (Zhou *et al.*, 2008).

Stem cell technology will also become invaluable in animal science, and perhaps even animal conservation (Trounson, 2008). One exciting new direction currently underway is to generate iPS cells in endangered species, and to re-establish these populations through chimerism in closely-related species.

The stem cell revolution was initially delayed by funding restrictions, arising from those with ethical concerns about using human embryos for research. The tide is turning, however, not only because of wider acceptance of the technology and appreciation for its potential importance, but also because of iPS cell technology, which obviates the use of human embryos. As a result, many agencies around the world are now funding stem cell research, and growing numbers of scientists and their students are entering the field. The result should be a global collaboration focused on delivering clinical outcomes of immense benefit to the world's population. We are just at the beginning of a very long road of work and discovery, but one thing is certain: stem cell research is vital and must go forward.

Alan Trounson
California Institute for Regenerative Medicine
San Francisco, CA, USA

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A New Path: Induced Pluripotent Stem Cells

Embryonic stem (ES) cells proliferate rapidly while maintaining pluripotency, namely, the ability to differentiate into all cell types. Human ES cells have been considered promising sources in cell transplantation therapies for various diseases and injuries, such as spinal cord injury, myocardial infarction, type I diabetes, and muscular dystrophy. The clinical application of human ES cells, however, faces difficulties regarding the use of human embryos, as well as tissue rejection following implantation. One way to circumvent these obstacles is to generate pluripotent stem cells directly from somatic cells. To this end, it is necessary to identify the factors that induce pluripotency in somatic cells. In 1960, Gurdon and his colleagues generated tadpoles by transferring the nuclei of intestinal cells from an adult frog into oocytes (Gurdon and Byrne, 2003). His successful cloning showed that pluripotency-inducing factors do indeed exist.

In 2006, induced pluripotent stem (iPS) cells were generated from mouse embryonic or adult fibroblasts by the retrovirus-mediated introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). These iPS cells are similar to ES cells in morphology, proliferation, and teratoma formation. Furthermore, mouse iPS cells were proved to be competent for adult chimeric mice and germline transmission. More recently iPS cells have been generated without Myc retroviruses. In addition to fibroblasts, iPS cells have been generated from mouse hepatocytes, epithelial cells, pancreatic cells, neural stem cells, and B-lymphocytes. These data demonstrated that pluripotency can be induced in various somatic cells, using only a few defined factors.

It is still not known how iPS cells can be generated from somatic cells by just a few transcription factors (Yamanaka, 2007; Jaenisch and Young, 2008). Oct3/4, Sox2, and Klf4 synergistically regulate the expression of pluripotency-associated genes, while suppressing lineage-specific genes in ES cells. They cannot access the target genes in somatic cells, because of the epigenetic modification of chromatin, and the presence of transcriptional suppressors. It is likely that these blockers cause the efficiency of iPS cell generation to be extremely low. The mystery which remains to be solved is precisely how these three transcription factors override various obstacles and reach the target genes in a small portion of transduced cells. Stochastic events may play a role in this phenomenon. Alternatively, a more sophisticated scenario may be involved in such transcription factor-mediated direct reprogramming.

In 2007, iPS cells were established from human fibroblasts by the introduction of the same four factors (Takahashi et al., 2007), or a slightly different combination (Yu et al., 2007). Because of the substantial differences between mouse and human ES cells, it was not known whether the same factors which we had identified in mice could also generate human iPS cells. However, it proved to be surprisingly easy to generate human iPS cells with the same four factors. Many other laboratories have now also generated human iPS cells. This rapid progress is attributable to the numerous recent findings obtained from human ES cell research. The culture requirements of human iPS cells and their appearance have been well characterized. Without this knowledge, human iPS cells could not have been generated so rapidly.

Human iPS cells are similar to human ES cells in many aspects. They form tightly-packed and flat colonies. Each cell exhibited morphology similar to that of human ES cells, characterized by large nuclei and scant cytoplasm. They expressed hES cell-specific surface antigens, including SSEAs, TRAs, and NANOG protein. Human iPS cells also had growth potential, gene expression patterns, telomerase activity, and an epigenetic status similar to those observed in human ES cells. Human iPS cells could differentiate into three germ layers through embryoid bodies and in teratomas. In addition, they have also been differentiated directly into neurons and beating cardiomyocytes *in vitro*. These data demonstrated that iPS cells can be generated not only from mouse, but also from human fibroblast cultures with the same defined factors.

In 2008, two groups succeeded in generating iPS cells from various patients, including those suffering from amyotrophic lateral sclerosis (ALS), Parkinson's disease, muscular dystrophy, and type I diabetes (Dimos et al., 2008; Park et al., 2008). These cells could provide unprecedented opportunities to understand how these diseases develop, to screen effective drugs, and to predict both side-effects and toxicity. One of the challenges in these kinds of *in vitro* applications is the establishment of methods to recapitulate the pathogenesis in somatic cells derived from the patients' own iPS cells. This might be more difficult than anticipated in diseases like ALS, in which it takes 10 or more years before patients develop symptoms.

Another future application of the iPS cells technology is in the field of regenerative medicine. Patient-specific iPS cells may make it possible to perform cell transplant therapy free from immune rejection. The banking of iPS

cells and differentiated cell progenies of various HLA types might be an alternative way to carry out regenerative medicine successfully (Nakatsuji et al., 2008). However, iPS cell technology still faces various safety issues that must be overcome prior to clinical application. The generation of iPS cells requires the dedifferentiation and reactivation of cell cycles. The same two phenomena take place during tumorigenesis. Although the recent demonstration of iPS cell generation by integration-free methods is an important step toward the clinical application, extensive basic research is still required to assure the safety of such iPS cells.

iPS cell technology is still in its infant stage (Nishikawa et al., 2008). There are many obstacles, as well as expectations. The molecular mechanisms should be thoroughly elucidated. The safety of using this technology should be extensively characterized. Effective protocols for directed differentiation into various lineages should be established. It is very difficult to predict where the technology will be five years from now. More and more talented scientists are getting involved in iPS cell research. I believe fair, but intense, competition among multiple laboratories will speed up the progress of the field. Breakthroughs will occur, which will boost the advancement of this field. In humans, it takes 20 years for infants become adults. iPS cells technology, although in its infant stage, may take a shorter time to mature and be ready for various clinical applications.

Shinya Yamanaka
Center for iPS Cell Research & Application, Kyoto University, Japan
Gladstone Institute of Cardiovascular Disease, San Francisco

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"Stemness": Definitions, Criteria, and Standards

INTRODUCTION

Stem cells have recently generated more public and professional interest than almost any other topic in biology. One reason stem cells capture the imagination of so many is the promise that understanding their unique properties may provide deep insights into the biology of cells as well as a path toward treatments for a variety of degenerative illnesses. And although the field of stem cell biology has grown rapidly, there exists considerable confusion and disagreement as to the nature of stem cells. This confusion can be partly attributed to the sometimes idiosyncratic terms and definitions used to describe stem cells. Although definitions can be restrictive, they are useful when they provide a basis for mutual understanding and experimental standardization. With this intention, I present explanations of definitions, criteria, and standards for stem cells. Moreover, I highlight a central question in stem cell biology, namely the origin of these cells. I also suggest criteria or standards for identifying, isolating, and characterizing stem cells. Finally, I summarize the notion of "stemness" and describe its possible application in understanding stem cells and their biology.

WHAT IS A STEM CELL?

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells (Weissman *et al.*, 2001; Smith, 2001). More explicitly, stem cells can generate daughter cells identical to their mother (self-renewal) as well as produce progeny with more restricted potential (differentiated cells). This simple and broad definition may be satisfactory for embryonic or fetal stem cells that do not perdure for the lifetime of an organism. But this definition breaks down in trying to discriminate between transient adult progenitor cells that have a reduced capacity for self-renewal and adult stem cells. It is therefore important when describing adult stem cells to further restrict this definition to cells that self-renew throughout the life span of the animal (van der Kooy and Weiss, 2000). Another parameter that should be considered is potency: Does the stem cell generate to multiple differentiated cell types (multipotent), or is it only capable of producing one type of differentiated cell (unipotent)? Thus, a more complete description of a stem cell includes a consideration of replication capacity, clonality, and potency. Some theoretical as well as practical considerations surrounding these concepts are considered in this chapter.

Self-renewal

Stem cell literature is replete with terms such as "immortal," "unlimited," "continuous," and "capable of extensive proliferation," all used to describe the cell's replicative capacity. These rather extreme and vague terms are not very helpful, as it can be noted that experiments designed to test the "immortality" of a stem cell would by necessity outlast authors and readers alike. Most somatic cells cultured *in vitro* display a finite number of (less than 80) population doublings prior to replicative arrest or senescence, and this can be contrasted with the seemingly unlimited proliferative capacity of stem cells in culture (Houck *et al.*, 1971; Hayflick, 1973; Hayflick, 1974; Sherr and DePinho, 2000; Shay and Wright, 2000). Therefore, it is reasonable to say that a cell that can undergo more than twice this number of population doublings (160) without oncogenic transformation can be termed "capable of extensive proliferation." In a few cases, this criteria has been met, most notably with embryonic stem (ES) cells derived from either humans or mice as well as with adult neural stem cells (NSCs) (Smith, 2001; Morrison *et al.*, 1997). An incomplete understanding of the factors required for self-renewal *ex vivo* for many adult stem cells precludes establishing similar proliferative limits *in vitro*. In some cases, a rigorous assessment of the capacity for self-renewal of certain adult stem cells can be obtained by single-cell or serial transfer into acceptable hosts, an excellent example of which is adult hematopoietic stem cells (HSCs) (Allsopp and Weissman, 2002; Iscove and Nawa, 1997). Adult stem cells are probably still best defined *in vivo*, where they must display sufficient proliferative capacity to last the lifetime of the animal. Terms such as "immortal" and "unlimited" are probably best used sparingly if at all.

Clonality

A second parameter, perhaps the most important, is the idea that stem cells are clonogenic entities: single cells with the capacity to create more stem cells. This issue has been exhaustively dealt with elsewhere and is essential for any definitive characterization of self-renewal, potential, and lineage.[1] Methods for tracing the lineage of stem cells are described in subsequent chapters. Although the clonal "gold standard" is well understood, there remain several confusing practical issues. For instance, what constitutes a cell line? The lowest standard would include any population of

cells that can be grown in culture, frozen, thawed, and subsequently repassaged *in vitro.* A higher standard would be a clonal or apparently homogenous population of cells with these characteristics, but it must be recognized that cellular preparations that do not derive from a single cell may be a mixed population containing stem cells and a separate population of "supportive" cells required for the propagation of the purported stem cells. Hence, any reference to a stem cell line should be made with an explanation of their derivation. For example, it can be misleading to report on stem cells or "stem cell lines" from a tissue if they are cellular preparations containing of a mixed population, possibly contaminated by stem cells from another tissue.

Potency

The issue of potency maybe the most contentious part of a widely accepted definition for stem cells. A multipotent stem cell sits atop a lineage hierarchy and can generate multiple types of differentiated cells, the latter being cells with distinct morphologies and gene expression patterns. At the same time, many would argue that a self-renewing cell that can only produce one type of differentiated descendant is nonetheless a stem cell (Slack, 2000). A case can be made, for clarity, that a unipotent cell is probably best described as a progenitor. Progenitors are typically the descendants of stem cells, only they more constrained in their differentiation potential or capacity for self-renewal and are often more limited in both senses.

Definition

In conclusion, a working definition of a stem cell is a clonal, self-renewing entity that is multipotent and thus can generate several differentiated cell types. Admittedly, this definition is not applicable in all instances and is best used as a guide to help describe cellular attributes.

WHERE DO STEM CELLS COME FROM?

The origin or lineage of stem cells is well understood for ES cells; their origin in adults is less clear and in some cases controversial. It may be significant that ES cells originate before germ layer commitment, raising the intriguing possibility that this may be a mechanism for the development of multipotent stem cells, including some adult stem cells. The paucity of information on the developmental origins of adult stems cells leaves open the possibility that they too escape lineage restriction in the early embryo and subsequently colonize specialized niches, which function to both maintain their potency as well as restrict their lineage potential. Alternatively, the more widely believed, though still unsubstantiated, model for the origin of adult stem cells assumes that they are derived after somatic lineage specification, whereupon multipotent stem cells–progenitors arise and colonize their respective cellular niches. In this section, I briefly summarize the origin of stem cells from the early embryo and explain what is known about the ontogeny of adult stem cells focusing attention on HSCs and NSCs.

Stem Cells of the Early Embryo

Mouse and human ES cells are derived directly from the inner cell mass of preimplantation embryos after the formation of a cystic blastocyst (Papaioannou, 2001). This population of cells would normally produce the epiblast and eventually all adult tissues, which may help to explain the developmental plasticity exhibited by ES cells. In fact, ES cells appear to be the *in vitro* equivalent of the epiblast, as they have the capacity to contribute to all somatic lineages and in mice to produce germ line chimeras. By the time the zygote has reached the blastocyst stage, the developmental potential of certain cells has been restricted. The outer cells of the embryo have begun to differentiate to form trophectoderm, from which a population of embryonic trophoblast stem cells has also been derived in mice (Tanaka *et al.*, 1998). These specialized cells can generate all cell types of the trophectoderm lineage, including differentiated giant trophoblast cells. At the egg cylinder stage of embryonic development (embryonic day (E) 6.5 in mice), a population of cells near the epiblast can be identified as primordial germ cells (PGCs), which are subsequently excluded from somatic specification or restriction (Saitou *et al.*, 2002). PGCs migrate to and colonize the genital ridges, where they produce mature germ cells and generate functional adult gametes. PGCs can be isolated either prior or subsequent to their arrival in the genital ridges and, when cultured with appropriate factors *in vitro*, can generate embryonic germ (EG) cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992). EG cells have many of the characteristics of ES cells with respect to their differentiation potential and their contribution to the germ line of chimeric mice (Labosky *et al.*, 1994; Stewart *et al.*, 1994). The most notable difference between ES and EG cells is that the latter may display (depending upon the developmental stage of their derivation) considerable imprinting of specific genes (Surani, 1998; Sorani, 2001; Howell *et al.*, 2001). Consequently, certain EG cell lines are incapable of producing normal chimeric mice.

Importantly, no totipotent stem cell has been isolated from the early embryo. ES and EG cells generate all somatic lineages as well as germ cells but rarely if ever contribute to the trophectoderm, extraembryonic endoderm, or extraembryonic mesoderm. Trophectoderm stem (TS) cells have been isolated, and these only generate cells of the trophectoderm lineage. It remains to be seen whether cells can be derived and maintained from totipotent embryonic stages.

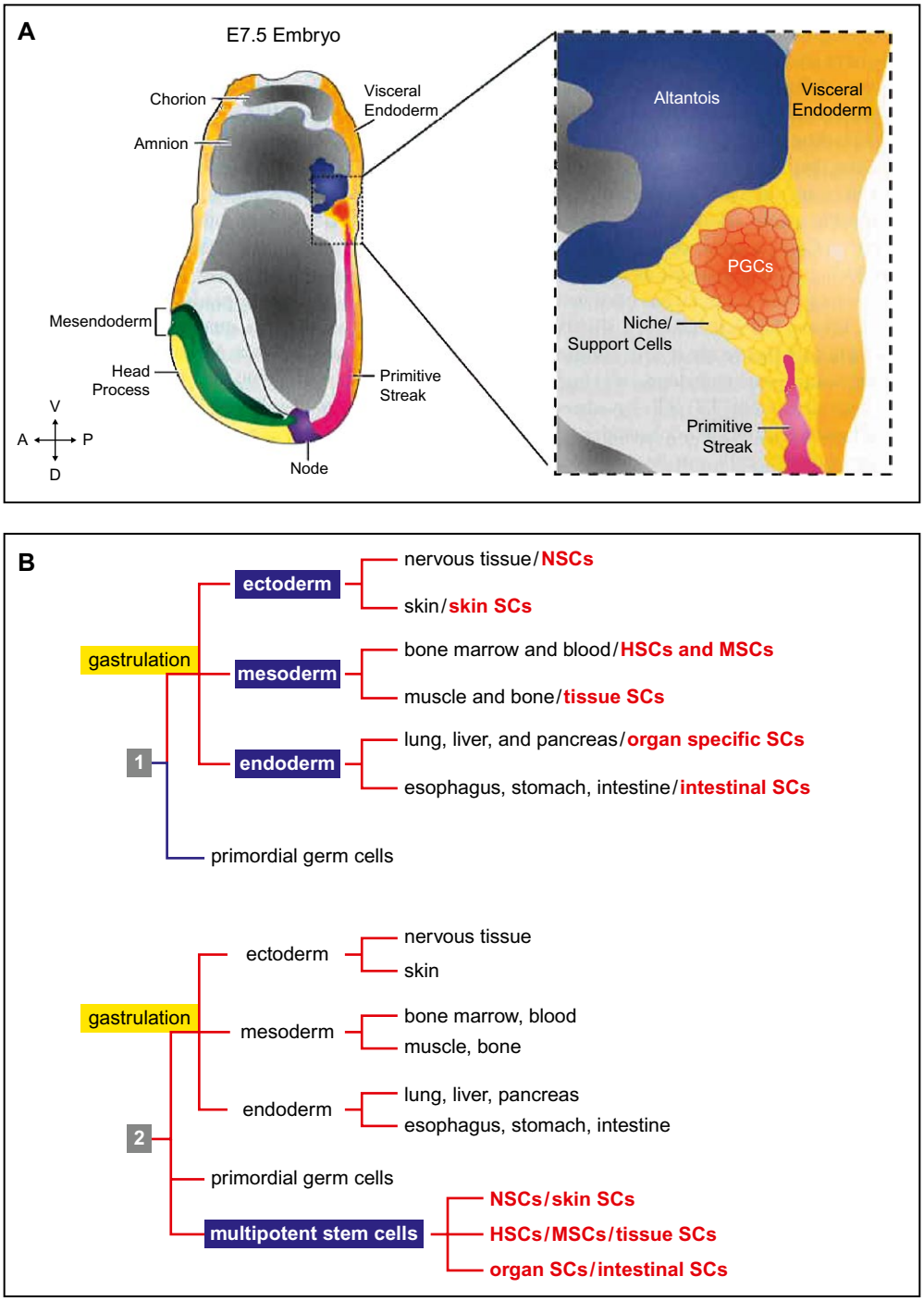


FIGURE 1 (A) Development of primordial germ cells. A schematic of an embryonic day 7.5 mouse embryo highlights the position of the developing primordial germ cells (PGCs) proximal to the epiblast. The expanded view on the right serves to illustrate the point that PGCs escape lineage commitment/restriction by avoiding the morphogenetic effects of migrating through the primitive streak during gastrulation. (B) Putative developmental ontogeny of stem cells. In lineage tree 1, the development of stem cells occurs after the formation of germ layers. These stem cells are thus restricted by germ layer commitment to their respective lineage (e.g., mesoderm is formed, giving rise to hematopoietic progenitors that become hematopoietic stem cells). Lineage tree 2 illustrates the idea that stem cells might develop similarly to PGCs, in that they avoid the lineage commitments during gastrulation and subsequently migrate to specific tissue and organ niches.

Although our understanding of cell fates in the early embryo is incomplete, it appears that the only pluripotent stem cells found after gastrulation are PGCs (with the possible exceptions of multipotential adult progenitor cells (Jiang *et al.*, 2002) and teratocarcinomas). It may be that PGCs escape germ layer commitment during gastrulation by developing near the epiblast and subsequently migrate to positions inside the embryo proper. This developmental strategy may

not be unique to PGCs, and it raises the interesting possibility that other stem cells might have similar developmental origins. Alternatively, it may be the case that adult stem cells are derived from PGCs. Although intriguing, it is important to stress that this idea lacks experimental evidence.

Ontogeny of Adult Stem Cells

The origin of most adult stem cells is poorly understood. With the issue of adult stem cell plasticity at the forefront, as described in this section, studies designed to elucidate the ontogeny of adult stem cells may help to reveal their specific lineage relationships and shed light on their plasticity and potential. Information on the origins of adult stem cells would also help to define the molecular programs involved in lineage determination, which may in turn provide insights into methods for manipulating their differentiation. To this end, I summarize what is known about the development of adult stem cells within the context of the hematopoietic and neural systems.

The development of hematopoietic cells in mice occurs soon after gastrulation (E7.5), although HSCs with the same activities as those in the adult have only been observed and isolated at midgestational stages (E10.5) (Orkin, 1996; Dzierzak, 2002; Weissman, 2000). These observations suggest that the embryo has a unique hematopoietic lineage hierarchy, which may not be founded by an adulttype HSC. Thus, hematopoiesis appears to occur at multiple times or in successive waves within the embryo, and the emergence of an HSC may not precede or be concomitant with the appearance of differentiated hematopoietic cells.

The first site of hematopoiesis in the mouse is the extraembryonic yolk sac, soon followed by the intraembryonic aorta–gonad–mesonephros (AGM) region. Which of these sites leads to the generation of the adult hematopoietic system and, importantly, HSCs is still unclear. Results from nonmammalian embryo-grafting experiments, with various findings in the mouse, suggest that the mammalian embryo, specifically the AGM, generates the adult hematopoietic system and HSCs (Kau and Turpen, 1983; Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996). Interestingly, the midgestational AGM is also the region that harbors migrating PGCs and is thought to produce populations of mesenchymal stem cells, vascular progenitors, and perhaps hemangioblasts (Molyneaux *et al.*, 2001; Minasi *et al.*, 2002; Alessandri *et al.*, 2001; Hara *et al.*, 1999; Munoz-Chapuli *et al.*, 1999). In the absence of studies designed to clonally evaluate the lineage potential of cells from the AGM, and without similarly accurate fate mapping of this region, it remains possible that all of the adult stem cell types thought to emerge within the AGM arise from a common unrestricted precursor. This hypothetical precursor could help to explain reports of nonfusion-based adult stem cell plasticity. The observed lineage specificity of most adult stem cells could likewise be attributed to the high-fidelity lineage restriction imposed on them by the specific niche they colonize or are derived from. Simple ideas such as these have not been ruled out by experimental evidence, underscoring both the opportunity and the necessity for further study of the developmental origins of adult stem cells.

A key lesson from studies of the developing hematopoietic system is that the appearance of differentiated cells does not tell us where or when the corresponding adult stem cells originate. Definitive lineage tracing, with assays of clonogenic potential, remains the method of choice for identifying the origin of stem cells. Another potential pitfall revealed by these studies is that the definition of the stem cell can make all the difference in its identification.

The development of NSCs begins with the formation of nervous tissue from embryonic ectoderm following gastrulation. Induction of the neural plate is thought to coincide with the appearance of NSCs as well as restricted progenitor types (Temple, 2001). The exact frequency and location of stem cells within the developing neuroepithelium remains unknown; specific markers must be discovered to fully unravel this question. An emerging view in the field is that embryonic neuroepithelia generate radial glial that subsequently develop into periventricular astrocytes and that these cells are the embryonic and adult NSCs within the central nervous system (Alvarez-Buylla *et al.*, 2001; Tramontin, 2003; Doetsch *et al.*, 1999; Gaiano and Fishell, 2002). Developing and adult NSCs also appear to acquire positional and temporal information. For example, stem cells isolated from different neural regions generate region-appropriate progeny (Kalyani *et al.*, 1998; He *et al.*, 2001; Anderson *et al.*, 1997). In addition, several studies suggest that temporal information is encoded within NSCs, that earlier stem cells give rise more frequently to neurons, and that more mature stem cells preferentially differentiate into glia (Temple, 2001; Qian *et al.*, 2000; White *et al.*, 2001). Moreover, more mature NSCs appear incapable of making cells appropriate for younger stages when transplanted into the early cerebral cortex (Desai and McConnell, 2000). Thus, the nervous system appears to follow a classical lineage hierarchy, with a common progenitor cell generating most if not all differentiated cell types in a regional- and temporal-specific manner. There may also be rare stem cells in the nervous system, perhaps not of neural origin, that have greater plasticity in terms of producing diverse somatic cell types and lacking temporal and spatial constraints (Weissman, 2000; Temple, 2001). There are several caveats that must be considered when describing the developmental origins of NSCs. First, disrupting the neuroepithelia to purify NSCs may have the undesirable effect of dysregulating spatial patterning acquired by these cells. Second, growth of purified NSCs in culture may reprogram the stem cells through exposure to nonphysiological *in vitro* culture conditions. Both of

these problems can be addressed either by *in vivo* lineage tracing or by prospectively isolating NSCs and transplanting them into acceptable hosts without intervening culture. Carefully designed experiments promise to answer questions important not only for stem cell biology but also for neuroembryology and development. These include which features of the developmental program are intrinsic to individual cells, which differentiation or patterning signals act exclusively to instruct specific cell fates, and how developmental changes in cell-intrinsic programs restrict the responses of progenitors to cell-extrinsic signals.

HOW ARE STEM CELLS IDENTIFIED, ISOLATED, AND CHARACTERIZED?

How stem cells are identified, isolated, and characterized are the key methodological questions in stem cell biology, so much so that subsequent chapters are devoted to addressing these problems in detail. Here, I briefly outline standards and criteria that may be employed when approaching the challenge of identifying, isolating, and characterizing a stem cell.

Embryonic Stem Cells

The basic characteristics of an ES cell include self-renewal, multilineage differentiation *in vitro* and *in vivo*, clonogenicity, a normal karyotype, extensive proliferation *in vitro* under welldefined culture conditions, and the ability to be frozen and thawed. In animal species, *in vivo* differentiation can be assessed rigorously by the ability of ES cells to contribute to all somatic lineages and produce germ line chimerism. These criteria are not appropriate for human ES cells; consequently, these cells must generate embryoid bodies and teratomas containing differentiated cells of all three germ layers. Moreover, as a stringent *in vivo* assessment of pluripotency is impossible, human ES cells must be shown to be positive for well-known molecular markers of pluripotent cells. These markers are defined as factors expressed consistently, and enriched, in human ES cells (Brivanlou *et al.*, 2003). As a substitute for whole-animal chimerism, human ES cells could be tested for their contributions to specific tissues when transplanted in discrete regions of nonhuman adults or embryos. A complementary analysis might include transplanting human ES cells into nonhuman blastocysts and evaluating their contribution to various organs and tissues, though this experiment has raised ethical concerns in some quarters. Finally, a practical consideration is the passage number of ES cells. Although it is important to establish the capacity of ES cells to proliferate extensively, it is equally important that lowpassage cells are evaluated experimentally to guard against any artifacts introduced through *in vitro* manipulation.

Adult Stem Cells

The basic characteristics of an adult stem cell are a single cell (clonal) that self-renews and generates differentiated cells. The most rigorous assessment of these characteristics is to prospectively purify a population of cells (usually by cell surface markers), transplant a single cell into an acceptable host without any intervening *in vitro* culture, and observe selfrenewal and tissue, organ, or lineage reconstitution. Admittedly, this type of *in vivo* reconstitution assay is not well defined for many types of adult stem cells. Thus, it is important to arrive at an accurate functional definition for cells whose developmental potential is assessed *in vitro* only. Above all, clonal assays should be the standard by which fetal and adult stem cells are evaluated because this assay removes doubts about contamination with other cell types.

Two concepts about the fate or potential of stem cells have moved to the forefront of adult stem cell research. The first is plasticity, the idea that restrictions in cell fates are not permanent but are flexible and reversible. The most obvious and extreme example of reversing a committed cell fate comes from experiments in which a terminally differentiated somatic cell generates to another animal following nuclear transfer or cloning (Solter, 2000; Rideout *et al.*, 2001). Nuclear transfer experiments show that differentiated cells, given the appropriate conditions, can be returned to their most primal state. Thus, it may not be surprising if conditions are found for more committed or specified cells to dedifferentiate and gain a broader potential. A related concept is that of transdifferentiation. Transdifferentiation is the generation of functional cells of a tissue, organ, or lineage that is distinct from that of the founding stem cell (Liu and Rao, 2003; Blau *et al.*, 2001). Important issues here are whether the cells proposed to transdifferentiate are clonal and whether the mechanism by which they form the functional cell requires fusion (Medvinsky and Smith, 2003; Terada *et al.*, 2002; Wang *et al.*, 2003; Ying *et al.*, 2002). Experiments designed to carefully evaluate these possibilities will yield insight into the nature of stem cells.

STEMNESS: PROGRESS TOWARD A MOLECULAR DEFINITION OF STEM CELLS

Stemness refers to the common molecular processes underlying the core stem cell properties of self-renewal and the generation of differentiated progeny. Although stems cells in different cellular microenvironments or niches will by necessity have different physiological demands and therefore distinct molecular programs, there are likely certain genetic characteristics specific to and shared by all stem cells. Through transcriptional profiling, many of the genes enriched in ES cell, TS cell, HSC, and NSC populations have been identified (Ivanova *et al.*, 2002; Ramalho-Santos

et al., 2002; Tanaka *et al.*, 2002; Anisimov *et al.*, 2002; Luo *et al.*, 2002; Park *et al.*, 2002). By extending this approach to other stem cells and more organisms, it may be possible to develop a molecular fingerprint for stem cells. This fingerprint could be used as the basis for a molecular definition of stem cells that, when combined with their functional definition, would provide a more comprehensive set of criteria for understanding their unique biology. Perhaps more importantly, these types of studies could be used to help identify and isolate new stem cells. This goal is far from being accomplished, but the preliminary findings for specific stem cells have been described. The transcriptional profiling of stem cells has suggested that they share several distinct molecular characteristics. Stem cells appear to have the capacity to sense a broad range of growth factors and signaling molecules and to express many of the downstream signaling components involved in the transduction of these signals. Signal transduction pathways present and perhaps active in stem cells include TGF®, Notch, Wnt, and Jak/Stat family members. Stem cells also express many components involved in establishing their specialized cell cycles, either related to maintaining cell cycle arrest in G1 (for most quiescent adult stem cells) or connected to progression through cell cycle checkpoints promoting rapid cycling (as is the case for ES cells and mobilized adult stem cells) (Burdon *et al.*, 1999; Savatier *et al.*, 2002). Most stem cells also express molecules involved in telomere maintenance and display elevated levels of telomerase activity. There is also considerable evidence that stem cells have significantly remodeled chromatin acted upon by DNA methylases or transcriptional repressors of histone deacetylase and Groucho family members. Another common molecular feature is the expression of specialized posttranscriptional regulatory machinery regulated by RNA helicases of the Vasa type. Finally, a shared molecular and functional characteristic of stem cells appears to be their resistance to stress, mediated by multidrug resistance transporters, protein-folding machinery, ubiquitin, and detoxifier systems.

Although in its infancy, the search for a molecular signature to define stem cells continues. We have begun to understand in general terms what molecular components are most often associated with stem cells. In the future, it may be possible to precisely define stem cells as a whole and individually by their telltale molecular identities. Until that time, stemness remains a concept of limited utility with tremendous potential.

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Douglas A. Melton, PhD
Chad Cowen, PhD

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Part I

Introduction to Stem Cells

Chapter 1

Pluripotential Stem Cells from Vertebrate Embryos: Present Perspective and Future Challenges

Richard L. Gardner

INTRODUCTION

Many have contributed to the various developments that brought recognition of the enormous potential of cells of early embryonic origin for genetic modification of organisms, regenerative medicine, and in enabling investigation of facets of development that are difficult to explore *in vivo*. However, historically, this field is firmly rooted in the pioneering work of Roy Stevens and Barry Pierce on mouse teratomas and teratocarcinomas, tumors which continued for some time after these workers had embarked on their studies to be regarded with disdain by many mainstream pathologists and oncologists. While Stevens developed and exploited mouse strains with high incidences of such tumors to determine their cellular origins, Pierce focused his attention on the nature of the cell that endowed teratocarcinomas with the potential for indefinite growth which the more common teratomas lacked. Conversion of solid teratocarcinomas to an ascites form proved a significant advance in enabling dramatic enrichment of the morphologically undifferentiated cells in such tumors which their stem cells were expected to be included among. Then, in an experiment of heroic proportions, Kleinsmith and Pierce showed unequivocally that, on transplantation to histocompatible adult hosts, individual morphologically undifferentiated cells could form self-sustaining teratocarcinomas that contained as rich a variety of differentiated tissues as the parent tumor. Hence, the embryonal carcinoma (EC) cell, as the stem cell of teratocarcinomas has come to be known, was the first self-perpetuating pluripotential cell to be characterized. Though teratocarcinomas were obtained initially as a result of genetically-determined aberrations in the differentiation of male or female germ cells, it was found later that they could also be established in certain genotypes of mice by grafting early embryos ectopically in adults. Adaptation of culture conditions to enable EC cells to be perpetuated in an undifferentiated state or induced to differentiate *in vitro* soon followed. Although the range of differentiation detected in these circumstances was more limited than *in vivo*, it could nevertheless be quite impressive. Research on murine EC cells, in turn, provided the impetus for obtaining and harnessing the human counterpart of these cells from testicular tumors for *in vitro* study.

One outstanding question regarding the use of murine EC cells as a model system for studying aspects of development remained, namely the basis of their malignancy. Was this a consequence of genetic change or simply because such "embryonic" cells failed to relate to the ectopic sites into which they were transplanted? The obvious way of addressing this was to ask how EC cells behave when placed in an embryonic rather than an adult environment. This was done in three different laboratories by injecting the cells into blastocyst stage embryos. The results from each laboratory led to the same rather striking conclusion. EC cells which if injected into an adult, would grow progressively and kill it, were able to participate in entirely normal development following their introduction into the blastocyst. Using genetic differences between donor and host as cell markers, EC cells were found to be able to contribute to most if not all organs and tissue of the resulting offspring. Most intriguingly, according to reports from one laboratory, this could very exceptionally include the germline. The potential significance of this finding was considerable in terms of its implications for possible controlled genetic manipulation of the mammalian genome. This is because it raised the prospect of being able to select for very rare events, and thus bring the scope for genetic manipulation in mammals closer to that in microorganisms.

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There were problems, however. One was that the EC contribution in chimeric offspring was typically both more modest and more patchy than that of cells transplanted directly between blastocysts. The chimeras also not infrequently formed tumors, with those that proved to be teratocarcinomas often being evident already at birth. Therefore, it seems likely that regulation of growth of atleast some of the transplanted EC cells failed altogether. Other chimeras developed more specific tumors such as rhabdomyosarcomas as they aged which were also clearly of donor origin, thereby revealing that the transplanted EC cells had progressed further along various lineages before their differentiation went awry. In extreme cases the transplanted EC cells disrupted development altogether so that fetuses did not survive to birth. Although the best EC lines could give very widespread contributions throughout the body of chimeras, they did so only very exceptionally. Finally, the frequency with which colonization of the germ line could be obtained with EC cells was too low to enable them to be harnessed for genetic modification. It seemed likely, therefore, that the protracted process of generating teratocarcinomas *in vivo* and then adapting them to culture militated against retention of a normal genetic constitution by their stem cells. If this was indeed the case, the obvious way forward was to see if such stem cells could be obtained in a less circuitous manner. This prompted investigation of what happens when murine blastocysts are explanted directly on growth-inactivated feeder cells in an enriched culture medium. The result was the derivation of lines of cells that were indistinguishable from EC cells in both morphology and expression of various antigenic and other markers, as well as in the appearance of the colonies they formed during growth. Moreover, like EC cells, these self-perpetuating blastocyst-derived stem cells could form aggressive teratocarcinomas in both syngeneic and immunologically compromised non-syngeneic adult hosts. They differed from EC cells principally in giving much more frequent and widespread somatic chimerism following reintroduction into the preimplantation conceptus and, if tended carefully, also in routinely colonizing the germline. Moreover, when combined with host conceptuses whose development was compromised by tetraploidy, they could sometimes form offspring in which no host-derived cells were discernible. Thus, these cells, which exhibited all the desirable characteristics of EC cells and few of their shortcomings, came to be called embryonic stem (ES) cells. Once it had been shown that ES cells could retain their ability to colonize the germline after *in vitro* transfection and selection, their future was assured. Surprisingly, however, despite the wealth of studies demonstrating their capacity for differentiation *in vitro*, particularly in the mouse, the idea of harnessing ES cells for therapeutic purposes took a long time to take root. Thus, although Robert Edwards explicitly argued the case that human ES cells might be used thus more than 25 years ago, it is only within the past decade that this notion has gained momentum, encouraged particularly by derivation of the first cell lines from human blastocysts.

TERMINOLOGY

There is some confusion in the literature about terminology in discussing the range of different types of cells that ES cells are able to form, an attribute that, in embryological parlance, is termed their potency. Some refer to these cells as being totipotent in recognition of the fact that, at least in the mouse, they have been shown to be able to give rise to all types of fetal cells and, under certain conditions, entire offspring. However, this is inappropriate on two counts. First, totipotency is reserved by embryologists for cells that retain the capacity to form an entire conceptus, and thus give rise to a new individual, unaided. Apart from the fertilized egg, the only cells that have so far been shown to be able to do this are blastomeres from early cleavage stages. Second, murine ES cells seem unable to form all the different types of cell of which the conceptus is composed. Following injection into blastocysts, they normally give rise only to cell types that are products of the epiblast or fetal precursor cell lineage. While they can also form derivatives of the primitive endoderm lineage which, for some obscure reason they do much more readily *in vitro* than *in vivo*, they have never been convincingly shown to contribute to the trophectodermal lineage. Hence, a widely adopted convention is to describe ES cells as pluripotent stem cells, to distinguish them from stem cells like those of the hematopoietic system which have a narrower, but nevertheless impressive, range of differentiative potential. A further source of confusion is the surprisingly common practice of referring to cells, particularly putative ES cells from mammals other than the mouse, as totipotent because their nuclei have been shown to be able to support development to term when used for reproductive cloning.

Another facet of terminology relates to the definition of an ES cell, which again is not employed in a consistent manner. One view, to which the author subscribes, is that use of this term should be restricted to pluripotent cells derived from pre- or peri-implantation conceptuses that can form functional gametes, as well as the full range of somatic cells of offspring. While there are considerable differences between strains of mice in the facility with which morphologically undifferentiated cell lines can be obtained from their early conceptuses, competence to colonize the germline as well as somatic tissues seems nevertheless to be common to lines from all strains that have yielded them. This is true, for example, even for the non-obese diabetic (NOD) strain whose lines have so far been found to grow too poorly to enable their genetic modification.

ES-LIKE CELLS IN OTHER SPECIES

As shown in Table 1-1, cell lines that can be maintained for variable periods *in vitro* in a morphologically undifferentiated state have been obtained from morulae or blastocysts of a variety of species of mammals in addition to the mouse. They have also been obtained from the stage X blastoderms in the chick, and from blastulae in several different species of teleost fish. The criteria that have been employed to support claims that such lines are counterparts of murine ES cells are quite varied and, not infrequently, far from unequivocal. They range from maintenance of an undifferentiated morphology during propagation or expression of at least some ES cell markers, through differentiation into a variety of cell types *in vitro*, to production of histologically diverse teratomas or chimerism *in vivo*.

TABLE 1-1 Vertebrates Other Than Mouse From Which ES-like Cells Have Been Obtained

Species	Basis of Validation*
Rat	M&M & CP
Golden hamster	IVD
Rabbit	M&M, IVD & CP
Mink	M&M, IVD & T (with wide range of cell types in one study)
Dog	IVD
Cat	IVD
Pig	M&M, IVD & CP
Sheep	M&M & **
Cow	M&M, IVD & CP
Horse	IVD
Marmoset	IVD
Rhesus monkey	T
Human	T
Chicken	IVD & CP (including germline with low passage cells)
Medaka	IVD & CP
Zebra fish	IVD (limited) & CP (with short-term cultured cells)
Gilthead sea bream	IVD & (CP with short-term cultured cells)

**M & M = morphology and ES cell markers; IVD = differentiation* in vitro*; T = teratoma production* in vivo*; CP = chimaera production by morula aggregation or blastocyst injection.*
*** Exhibited an ES-like morphology initially but rapidly acquired a more epithelial one thereafter*

What such ES-like (ESL) cells lines have in common with murine ES cells, in addition to a morphologically undifferentiated appearance and expression of various genes associated with pluripotency, is a high nuclear/cytoplasmic ratio. Among the complications in assessing cell lines in different species is variability in morphology of the growing colonies. While colonies of ESL cells in the hamster and rabbit are very similar to those of murine ES cells, those of most other mammals are not. This is particularly true in the human whose undifferentiated ESL cell colonies closely resemble those formed by human EC cells of testicular origin, as also are those of ESL cells from other primates. In the marmoset, rhesus monkey, and human, ESL cells not only form relatively flattened colonies, but also exhibit a number of differences from mouse ES cells in the markers they express. Since they closely resemble human EC cells in all these respects, the differences were assumed until recently to relate to species rather than cell type.

In two studies in the sheep, colonies are reported to look like those formed by murine ES cells initially, but then to adopt a more epithelial-like appearance rapidly thereafter. This change in morphology bears an intriguing similarity to the transition in conditioned medium of murine ES to so-called epiblast-like (EPL) cells which is accompanied by loss of their ability to colonize the blastocyst. Given that this transition is said to be completely reversible, whether a comparable one is occurring spontaneously in sheep clearly warrants further investigation.

In no species has production of chimeras with ESL cells rivalled that obtained with murine ES cells. Where it has been attempted, both the rates and levels of chimerism are typically much lower than those found with murine ES cells. An apparent exception is one report for the pig, in which 72% of offspring were judged to be chimeric. However, this figure is presented in an overview of work that remains unpublished, and no details are provided regarding the number of times the donor cells had been passaged before being injected into blastocysts. In a subsequent study in this species using ES-like cells that had been through 11 passages, one chimera was recorded among 34 offspring. However, as the authors of this latter study point out, rates of chimerism of only 10–12% have been obtained following direct transfer of ICM cells in the pig. Hence, technical limitations may have contributed to the low success with ES-like cells in this species.

The only species listed in Table 1-1 in which colonization of the germline has been demonstrated are the chicken and the zebra fish, but in both cases this was with cells that had been passaged only 1–3 times before being injected into host embryos. Stem cells from early chick embryos that have been passaged for longer can give strong somatic chimerism, but have not yet been shown to be able to yield gametes. Consequently, in conformity with the terminology discussed earlier, morphologically undifferentiated cell lines in all species listed in Table 1-1 except the mouse

should be assigned the status of ES-like (ESL) cells rather than ES cells.

Generally, the strategy for attempting to derive ES cell lines in other species has been initially to follow more or less closely the conditions that proved successful in the mouse, namely the use of enriched medium in conjunction with growth-inactivated feeder cells and either leukemia inhibitory factor (LIF) or a related cytokine. Various modifications that have been introduced subsequently include same-species rather than murine feeder cells and, in a number of species including the human, dispensing with LIF. Optimal conditions for deriving cell lines may differ from those for maintaining them. Thus, in one study in the pig use of same-species feeder cells was found to be necessary to obtain cell lines, although murine STO cells were adequate for securing their propagation thereafter. Feeder-free conditions were found to work best in the case of both the medaka and the gilthead sea bream. Moreover, the cloning efficiency of human ESL lines was improved in serum-free culture conditions.

Unexpectedly, despite being closely related to the mouse, the rat has proved particularly refractory to derivation of ES cell lines (see Table 1-1). So far, the only cell lines that have proved to be sustainable in longer term in this species seem to lack all properties of mouse ES cells apart from colony morphology. Indeed, except for the 129 strain of mouse, establishing cells lines that can be propagated *in vitro* in a morphologically undifferentiated state seems almost more difficult in rodents than in most of the other vertebrates in which it has been attempted.

Overall, one is struck by species variability in the growth factors, status of conceptus or embryo, and other requirements for obtaining pluripotential cell lines in species other than the mouse. So far, one can discern no clear recipe for success. Of course, obtaining cells that retain the capacity to colonize the germline following long-term culture is essential only for the purpose of genetically-modifying animals in a controlled manner. Having cells that fall short of this but are nevertheless able to differentiate into a range of distinct types of cells *in vitro* may suffice for many other purposes, including regenerative medicine.

Recent Findings on Mouse Epiblast Cells

Recent findings in the mouse which may help to explain the differing experiences in other species emerged from attempts to derive ES cells from the epiblast of early post-implantation conceptuses. Stem cells exhibiting pluripotency could be obtained thus, but these clearly differed from true ES cells from pre-implantation conceptuses in conditions for their derivation and maintenance, their colony morphology, and also in how their differentiation was induced. Most interestingly, they not only resembled human ESL cells in these respects, but also almost entirely lacked the ability to yield chimerism following introduction into pre-implantation conceptuses. This raises the intriguing possibility that the mouse is peculiar in being permissive for derivation stem cells at an earlier stage in the epiblast lineage than other species. These novel pluripotential mouse cell lines have been termed "epiblast stem cells" or EpiSc.

EMBRYONIC GERM CELLS

The pre-implantation conceptus is not the only source of pluripotential stem cells in the mouse. Sustainable cultures of undifferentiated cells that resemble ES cells strikingly in their colony morphology have also been obtained from primordial germ cells and very early gonocytes in this species. These cells, termed embryonic germ (EG) cells, have also been shown to be capable of yielding high rates of both somatic and germline chimerism following injection into blastocysts.

The above findings have prompted those struggling to derive ES cell lines in other species to explore primordial germ cells as an alternative for achieving controlled genetic modification of the germ line. As shown in Table 1-2, EG-like (EGL) cells have been obtained in several mammals as well as the chick, but as with ES-like cells, their ability to participate in chimera-formation has, with one exception, only been demonstrated at low passage. Moreover, while donor cells have been detected in the gonad of a chimera obtained from low passage EG-like cells in the pig, no case of germline colonization has been reported except with cells from chick genital ridges that were cultured for only five days. Even here, the proportion of offspring of donor type was very low.

TABLE 1-2 Vertebrates From Which Embryonic Germ (EG) Cells Have Been Obtained

Species	Basis of validation *	Reference
Mouse	M&M	Resnick *et al.*
	CP	Matsui *et al.*
	CP (including germline)	Laborski *et al.*
	CP (including germline)	Stewart *et al.*
	IVD	Rohwedel *et al.*
Pig	CP	Shim *et al.*
	CP (with transfected cells)	Piedrahita *et al.*
Cow	IVD (& short-term CP)	Cherny *et al.*
Human	IVD	Shamblott *et al.*
Chicken	CP (including germline, but cells cultured for only five days)	Chang *et al.*
	CP	Park and Han

** Abbreviations as listed in the footnote to Table 1-1*

It is, however, noteworthy that even in the mouse rates of malformation and perinatal mortality appear to be higher in EG than in ES cell chimeras. This may relate to erasure of imprinting in the germ line which seems to have begun by the time primordial germ cells have colonized the genital ridges or, for certain genes, even earlier. It is perhaps because of such concerns that the potential of EG cells for transgenesis in strains of mice that have failed to yield ES cells has not been fully explored. Interestingly, unlike in the mouse, EGL cell lines derived from genital ridges and the associated mesentary of 5–11 week human fetuses seem not to have embarked on erasure of imprinting. Obviously, it is important to confirm that this is the case before contemplating use of such cells as grafts for repairing tissue damage in humans.

FUTURE CHALLENGES

The value of ES and ESL cells as resources for both basic and applied research is now acknowledged almost universally. Present barriers to exploitation of their full potential in both areas are considered below, together with possible ways of addressing these. Fundamental to progress is gaining a better understanding of the nature and the basic biology of these cells.

BIOLOGY OF ES AND ES-LIKE CELLS

Germline Competence

Although murine ES cells have been used very extensively for modifying the genome, there are still a number of problems that limit their usefulness in this respect. Among these is loss of competence to colonize the germline, a common and frustrating problem whose basis remains elusive. It is not attributable simply to the occurrence of sufficient chromosomal change to disrupt gametogenesis, because it can occur in lines and clones that are found to be karyotypically normal. At present, it is not known whether it is due to failure of the cells to be included in the pool of primordial germ cells, or their inability to undergo appropriate differentiation thereafter, possibly as a consequence of perturbation of the establishment of genomic imprinting or its erasure. Even within cloned ES lines, cells have been found to be heterogeneous with respect to expression of imprinted genes. Given that many ES cell lines are likely to have originated polyclonally from several epiblast founder cells, there is the further possibility that they might, *ab initio*, consist of a mixture of germline-competent and non-competent sub-populations. Recent studies on involvement of BMP signaling in the induction of primordial germ cells have been interpreted as evidence against a specific germ cell lineage in mammals. Particular significance has been attached to experiments in which distal epiblast, which does not usually produce primordial germ cells, was found to do so when grafted to the proximal site from whence these cells normally originate. However, because of the extraordinary degree of cell mixing that occurs in the epiblast before gastrulation, descendants of all epiblast founder cells are likely to be present throughout the tissue by the time of primordial germ cell induction. Hence, yet to be excluded is the possibility that competence for induction is lineage-dependent and thereby segregates only to some epiblast founders cells. Because ES cell lines are typically produced by pooling all colonies derived from a single blastocyst, they might therefore originate from of a mixture of germline-competent and non-competent epiblast founder cells.

Male ES cell lines have almost invariably been used in gene targeting studies, even though this complicates work on X-linked genes whose inactivation may lead to cell-autonomous early lethality or compromised viability in the hemizygous state. Here, female (XX) lines would, in principle, offer a simpler alternative, except they are generally held to suffer partial deletion or complete loss of one X-chromosome after relatively few passages. It is, however, not clear how secure this conclusion is, because few references to their use have appeared in literature since the early reports in which consistent loss of all or part of one X was first documented. More recently, one of only two female lines tested was found to be germline competent, but the entirely donor-derived litters were unusually small, raising the possibility not entertained by the authors that the line in question was XO. Interestingly, human ESL cell lines seem not to show a similar propensity for X-chromosome loss and, indeed, nor do murine EpiSc.

Origin and Properties of ES and ES-like Cells

It is evident from the earlier overview that there is considerable diversity even among eutherian mammals in the characteristics of cells from early conceptuses that can be perpetuated *in vitro* in a morphologically undifferentiated state. The reason for this is far from clear, particularly since the great majority of such cell lines have been derived at a corresponding stage, namely the pre-implantation blastocyst, often using inner cell mass tissue isolated from there. In the mouse, in contrast to their EC counterparts, ES cells have not been obtained from post-implantation stages, arguing that there is a rather narrow window during which their derivation is possible. What this relates to in developmental terms remains obscure, although the finding that ES cells can shift reversibly to a condition that shows altered colony morphology and gene expression, in conjunction with loss of ability to generate chimeras following blastocyst injection, offers a possible approach for addressing this problem. Whether the late blastocyst stage sets the limit for obtaining ESL cell lines has not yet been

addressed critically in other mammals. However, the possibility that ESL cells in other mammals correspond to murine EpiSc clearly warrants further consideration.

Just as ES cell lines have been obtained from preblastocyst stages in the mouse, so have ESL cell lines from other mammals. However, in the case of the mouse, ES cell lines derived from early cleavage stages and morulae have not been found to differ in properties or developmental potential from those obtained from blastocysts, implying that all originate at the same very early stage in differentiation of the epiblast lineage. Thus claims that lines isolated from morulae have an advantage over those from blastocysts in being able to produce trophoblast have not been substantiated. However, species- as opposed to stage-related differences in the ability of cell lines to produce trophoblast tissue have been encountered. Early claims that mouse ES cells can form trophoblastic giant cells are almost certainly attributable to short-term persistence of contaminating polar trophectoderm tissue. Thus, production of such cells seems to be limited to the early passage of ES lines derived from entire blastocysts. It has never been observed with lines established from microsurgically isolated epiblasts. While the situation is not clear in many other species, in primates differentiation of trophoblast has been observed routinely in ESL cell lines established from immunologically-isolated inner cell masses. Moreover, differentiation of human cell lines to the stage of syncytiotrophoblast-formation has been induced efficiently by exposing them to BMP4.

Pluripotency

A seminal characteristic of ES or ESL cells is their pluripotency. The most critical test of this, which is not practicable in some species, particularly the human, is the ability to form the entire complement of cells of normal offspring. This assay, which was originally developed in the mouse, entails introducing clusters of ES cells into conceptuses whose development has been compromised by making them tetraploid, either by suppressing cytokinesis or through fusing sister blastomeres electrically at the two-cell stage. ES cells are then either aggregated with the tetraploid cleavage stages or injected into tetraploid blastocysts. Some of the resulting offspring contain no discernible host cells. It seems most likely that host epiblast cells are present initially and play an essential role in "entraining" the donor ES cells before being outcompeted, since groups of ES cells on their own cannot substitute for the epiblast or inner cell mass (author's unpublished observations). Selection against tetraploid cells is already evident by the late blastocyst stage in chimeras made between diploid and tetraploid morulae. Aggregating ESL cells between pairs of tetraploid morulae has been tried in cattle, but resulted in their contributing only very modestly to fetuses and neonates.

The second most critical test is whether the cells yield widespread, if not ubiquitous, chimerism in offspring following introduction into the early conceptus, either by injection into standard blastocysts or aggregation with morulae. The third is the formation of teratomas in ectopic grafts to histocompatible or immunosuppressed adult hosts, since it is clear from earlier experience with murine and human EC cells that a wider range of differentiation can be obtained in these circumstances than *in vitro*. For such an assay to be incisive it is necessary to use clonal cell lines and thus ensure that the diversity of differentiation obtained originates from one type of stem cell rather than a medley of cells with more limited developmental potential. While teratoma formation has been demonstrated with clonal ESL in the human, this is not true for corresponding cell lines in other species. A note of caution regarding the use of teratomas for assessing pluripotency comes from workers who found that hepatocyte differentiation depended not only on site of inoculation of mouse ES cells, but also the status of the host. Thus, positive results were obtained with spleen rather than hind-limb grafts, and only when using immmunologically-compromised nude rather than syngeneic mice as hosts.

Conditions of Culture

ES and ESL cells are usually propagated in complex culture conditions that are poorly defined, by virtue of including both growth-inactivated feeder cells and serum. This complicates the task of determining the growth factor and other requirements necessary for their maintenance, as well as for inducing them to form specific types of differentiated cells. While differentiation of murine ES cells in a chemically-defined medium has been achieved, their maintenance under such conditions has not. Murine ES cells can be both derived and maintained independently of feeder cells providing a cytokine that signals via the gp 130 receptor is present in the medium. However, whether the relatively high incidence of early aneuploidy recorded in the two studies in which LIF was used throughout in place of feeders is significant or coincidental is not clear. It is important to resolve this in order to learn whether feeder cells serve any other function than acting as a source of LIF or a related cytokine. Production of extracellular matrix is one possibility. However, species and cell-type variability is also a factor here since LIF is not required for maintaining human ESL lines, whose cloning efficiency is actually improved by omission of serum, although feeder cells are. The norm has been to use murine feeder cells both for obtaining and perpetuating ESL cell lines in other mammals, including the human. Recently, however, there has been a move to use feeders of human origin for human ESL cells. This is a notable development since it would obviously not be acceptable to employ xenogeneic cells for growing human ESL cell

lines that were destined for therapeutic rather than laboratory use. The situation is somewhat confusing in the case of the pig since in one study, but not in others, porcine feeders were found to be necessary for deriving ESL cell lines which could then be perpetuated on murine STO cells thereafter. Moreover, among teleost fish, feeder-free conditions seem to be optimal for maintaining ESL cells in both the medaka and the sea bream, but possibly not in the zebrafish.

Susceptibility Versus Resistance to Derivation

A further area whose further investigation could be informative in facilitating the establishment of pluripotent stem cell lines in other species is the basis of susceptibility versus resistance to ES cell derivation in the mouse. Thus, while ES cell lines can be obtained very easily in 129 mice, and relatively so in C57BL/6 and a few additional strains, other genotypes have proved more resistant. Notable among the latter is the non-obese diabetic (NOD) strain from which, despite considerable effort, genetically-manipulatable lines have not yet been obtained. This is not simply related to susceptibility of this strain to insulin-dependent diabetes, because the ICR strain from which NOD was developed has proved to be equally refractory. However, refractoriness seems to be a recessive trait because excellent lines with high competence to colonize the germline have been obtained from [NOD $\times$ 129]F1 epiblasts. Moreover, this is not the only example where refractoriness has been overcome by inter-crossing. Interestingly, marked differences in permissiveness for ESL cell derivation have also been found among inbred strains of the medaka fish.

Human ES-like Cell

Mouse EC and ES cells have already been used extensively to study aspects of development which, for various reasons, are difficult to investigate in the intact conceptus. Exploiting corresponding cells for this purpose is even more pressing for gaining a better understanding of early development in our own species, given the relative scarcity of material, ethical concerns about experimenting on conceptuses, and statutory or technical limitations on the period for which they can be maintained *in vitro*. Obviously, in view of their provenance, human ESL cells are likely to provide a more apposite model system than human EC cells, which have mainly been used until recently. One concern here is that so-called "spare" conceptuses, i.e., those that are surplus to the needs of infertility treatment, are the sole source of material for producing human ESL cell lines. Since the conceptuses produced *in vitro* by IVF or related techniques that are judged to be of the highest quality are selected for treating infertility treatment, those used for deriving ESL lines tend to be of lower quality. Does this matter so far as the properties of the resulting cell lines are concerned, particularly if their use therapeutically is contemplated? Is the ability to form a blastocyst that looks satisfactory morphologically adequate, or will it prove acceptable to produce conceptuses specifically for generating ESL cell lines so that quality is less of a concern?

ES Cell Transgenesis

One important use of ES cell transgenesis is to obtain animal models of human genetic diseases. Since few would claim that the mouse is the ideal species for this purpose, the incentive for being able to undertake such studies in more appropriate or experimentally tractable mammals must remain a high priority. For example, given its widespread use for studying respiratory physiology, the sheep would obviously be a more relevant species than the mouse as a model system for studying cystic fibrosis. However, unless pluripotential cells that are able to colonize the germline can be obtained in other species, this particular approach to transgenesis will continue to be limited to the mouse. Although the feasibility of an alternative strategy, namely genetically modifying and selecting non-germline competent cells such as fetal fibroblasts and then exploiting transfer of their nuclei to oocytes has been demonstrated, it is extremely demanding technically and entails considerable fetal attrition.

STEM CELL THERAPY

Potential Hurdles

One of the major present interests in ESL cells is the prospect of exploiting them therapeutically to repair damage to tissues or organs resulting from disease or injury. This poses a host of new challenges, not all of which have so far received the attention they deserve. Perhaps the most obvious one is whether it will be possible efficiently to obtain directed differentiation of stem cells to yield pure cultures of the desired type of more differentiated cells, as opposed to a mixed population. If the latter proves to be the case, the rigorous purging of cultures of residual undifferentiated or inappropriately differentiated cells will be necessary. How this is approached will depend on whether any contamination of grafts is acceptable and, if so, how much. One way in which this particular problem has been circumvented in murine model systems for *in vitro* differentiation of ES cells is to transfect them with the coding region of a gene for an antibiotic resistance or fluorescent protein coupled to the promoter of a gene that is expressed only in the desired type of differentiated cell. Recent advances have made it possible to carry out similar genetic modification of human ESL cells. While very effective selection of the desired type of differentiated cell may be achieved with this approach, it remains to be seen whether use of genetically modified

cells will be acceptable in a clinical, as opposed to a laboratory, context.

A further important issue in contemplating stem cell therapy is the cycle status of the desired type of cell. In certain cases, including cells that are not post-mitotic in grafts may be highly undesirable or even hazardous. In others, the presence of such cells may be essential to meet the demands of tissue growth or turnover. The latter would depend on obtaining the differentiation of ESL cells to stem cells rather than fully differentiated cells of the desired type. Given the growing evidence that tissue stem cells require a specific niche for their maintenance, this could well prove difficult to achieve. Establishing and maintaining an appropriate niche *in vitro* so as to be able to enrich for tissue-specific stem cells is likely to pose a considerable challenge, and will unquestionably depend on better knowledge of the normal biology of individual tissues than we have at present.

Yet another important issue is whether engrafted cells will survive and function properly when placed in a damaged tissue or organ. In cases where the donor cells are to provide a hormone, neural transmitter, or soluble growth factor, it may be possible to place them at some distance from the site of damage. However, where this is not practicable, there remains the question of whether transplanted cells will fare any better than native ones in a tissue or organ that has been seriously damaged by disease or injury. If they do not, how can one circumvent this difficulty, bearing in mind that achieving complex organogenesis *in vitro* is still a rather remote prospect? Regarding neuro-degenerative disease, some progress has already been made in "cleaning up" sites of tissue damage. For example, antibody-mediated clearance of plaques from the brain in transgenic mice over-expressing amyloid precursor protein has been demonstrated. However, such intervention may not be necessary in all cases. Transplanting differentiated murine ES cells enriched for putative cardiomyocytes to a damaged region of the left ventricle in rats led concomitantly to a reduction in size of this region and to an improvement in the performance of the heart.

Significant progress with achieving limited organogenesis *in vitro* has been reported for the urinary bladder. Biopsies of this organ were obtained from patients in which it was defective, and the inner urothelial and outer muscle cells expanded separately in culture for seeding on the appropriate surfaces of a biodegradable hemi-bladder-shaped matrix. Following the suturing of such *in vitro*-produced hemi-bladders into the bladder, obvious improvement in its function was recorded. Obviously, orchestrating more complex organogenesis *in vitro* offers greater challenges for the future.

Therapeutic Cloning

Establishing ESL cell lines from blastocysts derived by nuclear replacement, so-called "therapeutic cloning," has been widely advocated as a way of tailoring grafts to individual patients, thereby circumventing the problem of graft rejection. While the feasibility of producing ES cells in this way has been demonstrated in the mouse, there is sharp division of opinion within the biomedical research community about whether such cells would be safe to use therapeutically. Particular concern centers on the normality of the donor genome regarding the epigenetic status of imprinted genes.

Embryonic Versus Adult Stem Cells

Concern about use of early human conceptuses as a source of stem cells had focused much attention on recent studies which suggest that so-called "adult stem cells" are much more versatile in their range of differentiation than has generally been supposed. There is continuing lively debate about interpretation of many of the findings, which certainly do not at present justify the common assertion that adult cells render the use of ESL cells for therapeutic purposes unnecessary. Of particular concern is a growing body of evidence that adult cells may not be changing their differentiated state as independent entities, but through fusing with cells of the type to which they are claimed to have converted.

There is a further, more general point that, with few exceptions, among which the hematopoietic system is the clearest example, evidence is lacking that cells from adult organs and tissues which can be propagated in culture actually functioned as stem cells *in situ*. Hence, adoption of the term "stem cell" for cells from many adult sources is questionable. It is possible, if not likely, that cells which are strictly post-mitotic in their normal environment can nevertheless be induced to resume cycling when removed from it and placed in an enriched culture medium which may contain growth factors to which they would not otherwise be exposed. Such cells might well lack features of true stem cells, such as accurate proofing of DNA replication, conservation of turnover through transit amplification of differentiating progeny, and maintenance of telomere length. They might therefore be severely compromised in their ability to function in grafts.

Recently, however, the distinction between adult and embryonic stem cells has become blurred as a result of the demonstration in both mouse and human that various types of adult somatic cells can be converted into pluripotent stem cells by transducing them with a small number of genes that are normally expressed in ES or ESL cells, respectively. Such induced pluripotential stem (iPS) cells offer a more realistic prospect than therapeutic cloning for producing specific patient-compatible tissue for grafting, providing the process of inducing them can be improved so as to avoid dependence on retroviral transduction of the requisite genes.

CONCLUSIONS

Since the pioneering studies of Steven and Pierce pointed the way in the 1950s and 1960s, impressive progress has been made in harnessing stem cells of embryonic, as opposed to fetal or adult, origin for both basic research and exploring new approaches to regenerative medicine. There is, however, still a great deal to be learnt about the origin and properties of such cells, as well as control of their self-renewal versus differentiation, if we are to take full advantage of what they have to offer. The effort of acquiring the necessary knowledge will undoubtedly provide us with the further reward of gaining deeper insight into the biology of stem cells in general.

KEY WORDS

Blastocyst The late pre-implantation stage of development when the embryo cavitates to form a hollow sphere bounded by a monolayer of trophectoderm, to part of the inner surface of which is attached a disk of cells called the inner mass cell. The former contributes exclusively to placental tissues and the latter both to the fetus and the placenta, plus additional extra-embryonic membranes.

Chimera An animal composed of cells originating from two or more embryos produced, for example, by injecting embryonic stem cells into a blastocyst of different genotype.

Embryonic stem cells (ES cells) Stem cells derived from the preimplantation embryo with an unrestricted capacity for self-renewal that has the potential to form all types of adult cells, including germ cells. Thus far, such cells have only be obtained from certain strains of mice.

Embryonic stem cell-like cells (ES-like cells) Stem cells that resemble murine embryonic stem cells, but whose potential to colonize the pre-implantation embryo and form all types of adults cells including germ cells has yet to be established.

Epiblast stem cells (EpiSc) Stem cells obtained from early postimplantation mouse embryos that resemble ESL cells from other species in terms of the conditions required for maintaining pluripotency and inducing differentiation.

Induced pluripotential stem cells (iPS cells) Differentiating or differentiated cells induced to return to a pluripotent state by manipulating them to express genes associated with pluripotency.

Pluripotency The potential to form most or all types of specialized cells: to be distinguished from totipotency, that is the ability to form a complete embryo capable of giving rise to offspring.

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Chapter 2

Embryonic Stem Cells in Perspective

Janet Rossant

Biologists have explored the development of embryos of all sorts, from worms to humans, in search of the answer to the question of how a complex organism derives from a single cell, the fertilized egg. We now know many of the genes involved in regulating development in different species and find remarkable conservation of genetic pathways across evolution. We also have a good understanding of the logic of development—how the embryo repeatedly uses the same kinds of strategies to achieve cellular specialization, tissue patterning, and organogenesis. One common strategy of development is the use of the stem cell to help generate and maintain a given tissue or organ. A stem cell is a cell that, when it divides, can produce a copy of itself, as well as a differentiated cell progeny. This self-renewal capacity underlies the ability of adult stem cells, such as hematopoietic stem cells and spermatogonial stem cells, to constantly renew tissues that turn over rapidly in the adult. The concept of the stem cell arose from the pioneering studies of Till and McCullogh on the hematopoietic stem cell and those of Leblond on spermatogenesis and the intestinal crypt. Even in tissues like the brain, where cells do not turn over so rapidly in the adult, there are long-lived quiescent stem cells that may be reactivated to repair damage.

Much current research is focused on the identification, characterization, and isolation of stem cells from the adult, with the hope that such cells may be useful for therapeutic repair of adult tissues either by exogenous cell therapy or by reactivation of endogenous stem cells. However, to date, most adult stem cells have restricted potential, and achieving indefinite proliferation and expansion of the stem cells in culture is still not routine. During embryogenesis, cells are initially proliferative and pluripotent; they only gradually become restricted to different cell fates. The question of whether pluripotent stem cells exist in the embryo has been of interest for years. In mammals, it was known in the 1960s and 1970s that early mouse embryos, up to late gastrulation stages, could produce tumors known as teratocarcinomas when transplanted to ectopic sites, such as the kidney capsule. These tumors contain a variety of differentiated cells types, including muscle, nerve, and skin, as well as an undifferentiated cell type, the embryonal carcinoma (EC) cell. EC cells could be propagated in the undifferentiated state *in vitro*. Importantly, Pierce in 1964 showed that a single EC cell could regenerate a tumor containing both EC cells and differentiated progeny (Kleinsmith and Pierce, 1964), demonstrating that EC cells are the stem cells of the tumor.

What was the relevance of these tumor cells to normal development? Many studies were carried out in the 1970s showing that EC cells could reveal their pluripotency when injected back into early embryos. The best, most karyotypically normal EC cells could contribute to many different cell types in the resulting chimeras, including, in rare instances, the germline. This led to excitement that these cells might be used to introduce new genetic alterations into the mouse and that normalization of tumorigenicity could be achieved by promoting differentiation of tumor cells. However, chimerism was often weak, and EC-derived tumors were a common feature of the chimeras (Papaioannouo and Rossant, 1984). Thus, although EC cells had remarkable properties of differentiation, they were still clearly tumor cells. In 1981, Martin (1981) and Evans and Kaufman (1981) discovered that permanent pluripotent cell lines, known as embryonic stem (ES) cells, could be derived directly from the blastocyst in culture. This changed the whole perspective of the field. The differentiation of these cells, although they make teratomatous tumors in ectopic sites, is easier to control than that of EC cells. Dramatically, ES cells grown for many passages in culture can make an entire mouse when supported by tetraploid extraembryonic tissues (Nagy *et al.*, 1993). When such mice are made from robust hybrid cell lines, they show no enhanced tumor susceptibility and appear normal in all respects (Eggan *et al.*, 2001). All of these properties have made mouse ES cells an incredibly powerful tool for introducing alterations into the mouse genome and analyzing their effects (Rossant and Nagy, 1995).

Are ES cells true stem cells? The *in vivo* equivalent of the ES cell is unclear. ES cells resemble the cells of the primitive ectoderm or epiblast in their gene expression patterns and their pattern of tissue contribution in chimeras. Transcription factors, such as Oct4 and Nanog, which are required for formation and survival of the pluripotent cells

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in the embryo, are also needed for ES survival (Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Nichols *et al.*, 1998). However, *in vivo*, the epiblast only has a limited period of possible stem cell expansion before all cells differentiate into the tissues of the three germ layers at gastrulation. Germ cells, which are set aside at gastrulation, go on to provide the gametes that will impart pluripotency to the zygotes of the next generation. However, there is no evidence that germ cells are a special stem cell pool in the epiblast, which could be the ES equivalent. Rather, it appears that all epiblast cells have the capacity to form germ cells in the right environment (Tam and Zhou, 1996). Thus, the germ cell is just one of the differentiation options of epiblast.

In vitro, it is clear that ES cells can be expanded indefinitely in the undifferentiated state, and still retain the capacity for differentiation. In this regard, they certainly have stem cell properties. However, there has not been a clear demonstration that a single cell can both self-renew and differentiate, as has been shown for EC cells. It is known that single cells are fully pluripotent, since chimeras made by injecting single ES cells into blastocysts show ES contributions to all fetal cell types analyzed (Beddington and Robertson, 1989). However, in some ways ES cells seem more like progenitor cells, where the population can be expanded by the right growth factor environment but all cells will differentiate when the supportive environment for self-renewal is removed. Practically, the difference is probably not important, but if true, it may be misleading to extrapolate from what we know about how ES cells maintain the proliferative state to other stem cells. The search for "stemness" genes and proteins may not be a useful undertaking until we agree on how to define different stem cell populations.

So much of the excitement about mouse ES cells has focused on their use as a tool for germline transmission of genetic alterations, that the remarkable differentiation properties of these cells in culture have been underexplored. The derivation of cell lines from early human embryos that seem to share many of the properties of mouse ES cells (Shamblott *et al.*; Thomson *et al.*, 1998) has refocused attention on the *in vitro* properties of ES cells. Many questions remain before ES cells can be transformed from an interesting biological system to a robust therapeutic modality for degenerative diseases. How similar are mouse and human ES cells, and how valid is it to use data from one to drive research in the other? How can ES cells be maintained through many passages in a truly stable state, where all cells are stem (or progenitor) cells, epigenetic programming is stable, and genetic abnormalities are minimal? How can ES cells be directed to differentiate reproducibly into given cell types, and how can differentiated progenitors be isolated and maintained? How can we ensure that ES cells will not be tumorigenic *in vivo?*

The recent discovery that expression of a small set of key transcription factors can reprogram adult differentiated cells to behave like ES cells—so-called induced pluripotent stem (iPS) cells—has galvanized the field of stem cell biology and its application to human disease. Ethical issues related to the use of human embryos for ES research are reduced and the possibility of generating patient-specific stem cells for future therapeutic applications becomes more realistic than previous suggested approaches using somatic cell nuclear transfer. At this stage, however, research on iPS cells is still in its infancy and the true nature of these cells and their therapeutic usefulness is unclear. The immediate opportunity presented by the iPS technology is to generate disease-specific iPS cells for studying human disease mechanisms in the Petri dish. In this application, as in ES applications in general, understanding how to reproducibly differentiate stem cells into mature specialized cell types will be key.

All this research on ES cells, both mouse and human, will provide new insights into embryonic development and new clues as to how to isolate and characterize new stem cells from different embryonic or adult tissues. Conversely, research on how normal embryonic development is regulated will provide new clues as to how to maintain and differentiate stem–progenitor cells in culture. The interplay between developmental biologists and stem cell biologists will be key to a fundamental understanding of stem cell development and its translation into therapeutic outcomes.

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Chapter 3

The Development of Epithelial Stem Cell Concepts

Christopher S. Potten and J. W. Wilson

INTRODUCTION

In the 1950s and 1960s, all proliferating cells in the renewing tissues of the body were regarded as having equal potential to self-maintain, one daughter cell on average from each division of a proliferative cell being retained within the proliferative compartment. Thus, all proliferating cells were regarded as stem cells. It proved somewhat difficult to displace this concept. Ground-breaking work by Till and McCulloch in 1961 provided the first clear evidence that for one of the replacing tissues of the body, the bone marrow, not all proliferative cells are identical. Their approach was to study the cells that were capable of repopulating hemopoietic tissues, following cellular depletion of the tissue by exposure to a cytotoxic agent, that is, radiation. Specifically, mice were irradiated to deplete their bone marrow of endogenous, functional hematopoietic precursors; then they were injected with bone marrow-derived precursors obtained from another animal. The exogenous cells were subject to a variety of treatments, prior to transplant. It was found that the hemopoietic precursors circulated in the host and seeded cells into various hemopoietic tissues, including the spleen. Those cells that seeded into the spleen and possessed extensive regenerative and differentiative potential grew by a process of clonal expansion to form macroscopically visible nodules of hemopoietic tissue, 10 to 14 days after transplant. By appropriate genetic or chromosome tracking (marking), it could be shown that these nodules were derived from single cells (i.e., they were clones) and that further clonogenic cells were produced within the clones. The colonies were referred to as spleen colonies, and the cells that form the colonies were called colony-forming units (spleen) (CFUs).

These experiments provided the theoretical basis for subsequent human bone marrow transplant studies. Through a variety of pre-irradiation manipulations and pre- and post-transplantation variables, this technique led to our current understanding of the bone marrow hierarchies or cell lineages and their stem cells. These studies showed that this tissue contained undifferentiated self-maintaining precursor cells that generated dependent lineages that were able to differentiate down a range of different pathways, generating a variety of cell types. Recent studies have suggested that these CFUs are not the ultimate hemopoietic stem cells, but are part of a stem cell hierarchy in the bone marrow.

Such clonal regeneration approaches have subsequently been developed for a variety of other tissues, notably by the imaginative approaches adopted by Rod Withers for epidermis, intestine, kidney, and testis. These clonal regeneration approaches were summarized and collected in a book produced in 1985, but the field was initiated by work done by Withers. These approaches implicated hierarchical organizations within the proliferative compartments of many tissues. The stringency of the criteria defining a clone varied enormously, depending as it did on the number of cell divisions required to produce the detectable clones. For epidermis and intestine, the stringency was high since the clones could be large and macroscopic, containing many cells resulting from many cell divisions. In fact, they were very similar in appearance to the spleen colony nodules.

One difficulty with the interpretation and generality of application to stem cell populations based on these clonal regeneration studies is the fact that, in order to see the regenerating clones, the tissue has to be disturbed, generally by exposure to a dose of radiation. This disturbance may alter the cellular hierarchies that one wishes to study and will certainly alter the nature (e.g., cell cycle status, responsiveness to signals, susceptibility to subsequent treatment) of the stem cell compartment. This has been referred to as the biological equivalent of the Heisenberg uncertainty principle in quantum physics. However, these clonal regeneration assays still provide a valuable and, in some places, unique opportunity to study some aspects of stem cell biology *in vivo*, that is, by using this approach to look at stem cell survival and functional competence under a variety of conditions.

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A DEFINITION OF STEM CELLS

Relatively few attempts have been made to define what is meant by the term stem cells, which has resulted in some confusion in the literature and the use of a variety of terms, the relationship between which sometimes remains obscure. These terms include precursors, progenitors, founder cells, and so on. The concept is further complicated by the use of terms such as committed precursors or progenitors and the sometimes confusing use or implication of the term differentiation. One difficulty in defining stem cells is the fact that the definitions are often very context-dependent and, hence, different criteria are brought into the definition by embryologists, hematologists, dermatologists, gastroenterologists, and other specialists.

In 1990 in a paper in *Development*, we attempted to define a stem cell. This definition was, admittedly, formulated within the context of the gastrointestinal epithelium, but we felt it had a broader application. The definition still largely holds and can be summarized as follows. Within adult replacing tissues of the body, the stem cells can be defined as a small sub-population of the proliferating compartment, consisting of relatively undifferentiated proliferative cells that maintain their population size when they divide, while at the same time producing progeny that enter a dividing transit population within which further rounds of cell division occur, together with differentiation events, resulting in the production of the various differentiated functional cells required of the tissue. The stem cells persist throughout the animal's lifetime in the tissue, dividing a large number of times; as a probable consequence of this large division potential, these cells are the most efficient repopulators of the tissue following injury. If this repopulation requires a re-establishment of the full stem cell compartment, the self-maintenance probability of the stem cells at division will be raised from the steady state value of 0.5 to a value between 0.5 and 1, which enables the stem cell population to be re-established, while at the same time maintaining the production of differentiated cells to ensure the functional integrity of the tissue.

The consequences of this definition are obvious, namely, that stem cells are:

- Rare cells in the tissue, vastly outnumbered by the dividing transit population, and are the cells upon which the entire lineage and ultimately the tissue are dependent.
- The only permanent long-term residents of the tissue.
- Cells at the origin of any cell lineages or migratory pathways that can be identified in the tissue.

The concept of differentiation enters into the definition of stem cells, and this, too, often leads to confusion. In our view, differentiation is a qualitative and relative phenomenon. Cells tend to be differentiated relative to other cells, and hence adult tissue stem cells may, or may not, be differentiated relative to embryonic stem cells (a point of current debate, bearing in mind the controversy in the literature concerning bone marrow stem cell plasticity). Stem cells produce progeny that may differentiate down a variety of pathways leading to the concept of totipotency and pluripotency of stem cells in terms of their differentiation. This is actually a strange concept to apply to a stem cell, since it is their progeny that differentiate and not the stem cells themselves. The fact that the progeny can differentiate down more than one lineage, as is very obviously the case in the bone marrow, results in bone marrow stem cells being referred to as pluripotent, and the initial dividing transit cells that initiate a lineage ultimately leading to specific differentiated cells can be thought of as committed precursors for that lineage.

Some of the instructive signals for differentiation in the hemopoietic cell lineage are now well understood, but such signals for other tissues organized on a cell lineage basis have yet to be determined. There is much debate in the literature concerning the extent to which stem cells may be instructed to produce progeny of specific differentiated types, and whether this is limited or unlimited. This topic is referred to as the degree of plasticity for stem cells. There are two very distinct issues here:

- The first is whether a stem cell, such as a bone marrow stem cell, is ever instructed by its environment in nature, or in laboratory or clinical situations, to make an apparently unrelated tissue cell type such as a liver, intestinal, or skin cell, and whether it can regenerate these tissues if they are injured. A subsidiary question is not whether this ever happens normally in nature, but whether we, as experimentalists or clinicians, can provide the necessary instructions or environment for this to happen in a controlled situation.
- The second issue relates not only to the stem cells, but also to the early progeny of stem cells from, for example, the bone marrow, and whether these cells that circulate around the body and may end up in a distant tissue can ultimately express differentiation markers unrelated to the bone marrow cell lineages, but specific to the tissue in which the cell then resides.

The former issue is one of the plasticity of the bone marrow stem cells, and the latter may be more an issue of the plasticity of the bone marrow-derived cell lineages. If a bone marrow stem cell can ever be instructed to be a gastrointestinal stem cell, it should be capable of undertaking all the functional duties of a gastrointestinal stem cell, including the regeneration of the gastrointestinal epithelium if it is subsequently injured. The cloning of animals by nuclear transfer technology into egg cytoplasm clearly demonstrates that all nuclei of the body contain a full complement of DNA, and that under the right environmental conditions this can be reprogrammed (or unmasked) by environmental signals to make all the tissues of the body. It should be remembered, however, that such cloning experiments, such as Dolly the

sheep, are rare and inefficiently produced events. They do, however, clearly indicate the enormous potential that can be achieved if we can provide the necessary instructive reprogramming signals. It should enable us in the future to reproducibly instruct any adult tissue stem cell to make any tissue of the body. If and when this becomes the case, the distinction between embryonic stem cells and adult tissue stem cells may disappear.

HIERARCHICALLY ORGANIZED STEM CELL POPULATIONS

The issue here is what determines the difference between a dividing transit cell and a stem cell, and whether that transition is an abrupt or a gradual one. One can think of this transition as being a differentiation event that distinguishes a dividing transit cell from a stem cell. This is an old argument. Do differentiation signals act on pre-existing stem cells, removing on average half the cells produced by previous symmetric divisions, or do the stem cells divide asymmetrically, to produce a differentiated progeny at division and a stem cell? One possibility is that this distinction is made at the time that a stem cell divides. Indeed, do they need to divide to differentiate? In this case, such divisions must be regarded as asymmetric, with the dividing stem cell producing one stem cell (i.e., for self-maintenance) and one dividing transit cell. This type of asymmetric division may occur in some tissues, such as the epidermis. If this is the case, however, the stem cell must also retain the potential to alter its self-maintenance probability, which for an asymmetric division is 0.5 in steady state, and adopt a value somewhat higher than this if stem cells are killed and require to be repopulated.

The current view regarding the bone marrow stem cells is that the transition between a stem cell and a dividing transit cell is a gradual one that occurs over a series of divisions within a cell lineage, which inevitably implies that one has a population of stem cells with a varying degree of stemness or, conversely, a varying degree of differentiation. For the bone marrow, one issue is whether experimentalists have ever identified the presence of the truly ancestral ultimate bone marrow stem cell. The difficulty here may be one of identifying and extracting such cells, the location of which is probably in the bone where they will be present in increasingly diminishing numbers, as one looks for the increasingly primitive cells.

Our current model for the gastrointestinal cellular organization, which is based on an attempt to accommodate as much experimental data as possible, is that the commitment to differentiation producing dividing transit cells does not occur at the level of the ultimate stem cell in the lineage, but at a position two or three generations along the cell lineage. If such a concept is drawn as a cell lineage diagram, the proliferative units in the intestine, the crypts, each contain four to six cell lineages and, hence, four to six lineage ancestor stem cells, but up to 30 second- and third-tier stem cells, which under steady state circumstances are inevitably displaced and moved toward the dividing transit compartment. But, if damage occurs in one or more of the ultimate stem cells, they can assume the mantle of the ultimate stem cell and repopulate the lineage. This gives rise to the concept of actual and potential stem cells (see Figure 3-1), which is discussed later in this chapter.

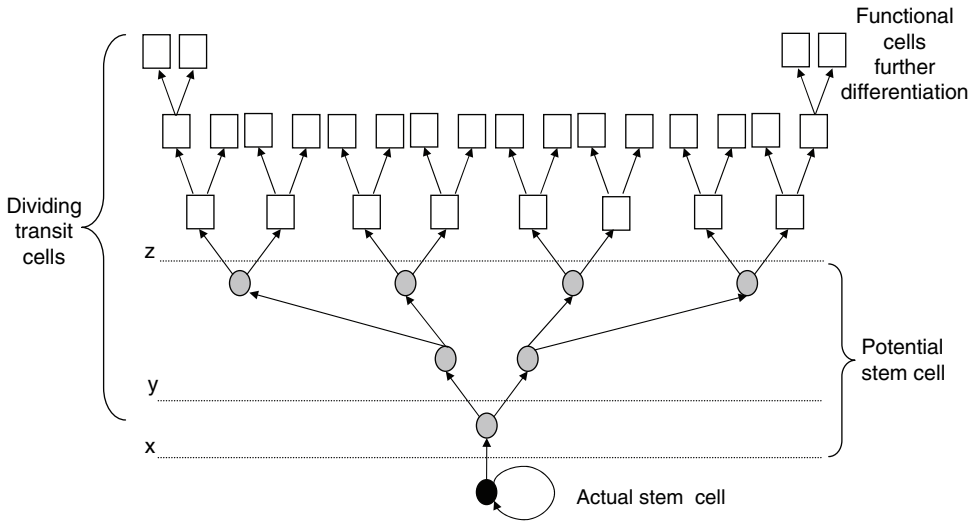


FIGURE 3-1 A typical stem cell-derived cell lineage that may be applicable to most epithelial tissues of the body. The lineage is characterized by a self-maintaining lineage ancestor actual stem cell (black) which divides and produces a progeny that enters a dividing transit population. The number of cell generations in the dividing transit population varies from tissue to tissue. The commitment to differentiation that separates the stem cell from the dividing transit population can occur at the point of the actual stem cell division (x), in which case the stem cells are dividing asymmetrical on average. This commitment may be delayed to point *y* or *z*, generating a population of potential stem cells that can replace the actual stem cell if it is killed. Under normal steady state circumstances, the potential stem cells form part of the dividing transit population and are gradually displaced down the lineage, undergoing further differentiation events if required to produce the functional mature cells of the tissue.

An analogy can be drawn here with the hierarchical organization within an organization such as the army, a concept that was discussed at the time we were formulating the text for the development paper in which we defined stem cells. In a military battlefield environment, the hierarchically organized army is under the control and ultimately dependent upon the highly-trained (or so one hopes) general. In the event that the general is killed in the battlefield, there may be a reasonably well-trained captain who can take over command and assume the insignia and uniform, as well as the function of the general. In the event that the captain, too, should be killed, there may be less well-trained officers who will attempt to assume the mantle of command. Ultimately, the vast majority of the troops, the privates, would be insufficiently trained or experienced to be able to adopt the functional role of the commander. However, the Dolly the sheep scenario suggests that occasionally a private, given a crash course in military strategy, might function as the officer-in-command. The analogy could be taken even further to relate to the apoptosis sensitivity that is seen in the gastrointestinal ultimate stem cells. These cells appear to adopt a strategy with complete intolerance to any genetic damage and a reluctance to undertake repair, since this may be associated with inherent genetic risk that they commit an altruistic suicide: the general who undergoes a nervous breakdown or serious injury and has to be removed from command.

In the small intestinal crypts, there have been no useful markers that permit the stem cells to be identified and, hence, studied. However, such markers are now being identified. In the absence of markers, the small intestine proved to be an invaluable biological model system to study stem cells because the cells of the intestinal cell lineage are arranged spatially along the long axis of the crypt. This can be demonstrated by cell migration tracking and mutational marker studies. As a consequence, the stem cells are known to be located at very specific positions in the tissue (crypts): the fourth-fifth cell position from the crypt base in the small intestine and at the very base of the crypt in the mid-colon of the large intestine (see Figure 3-2).

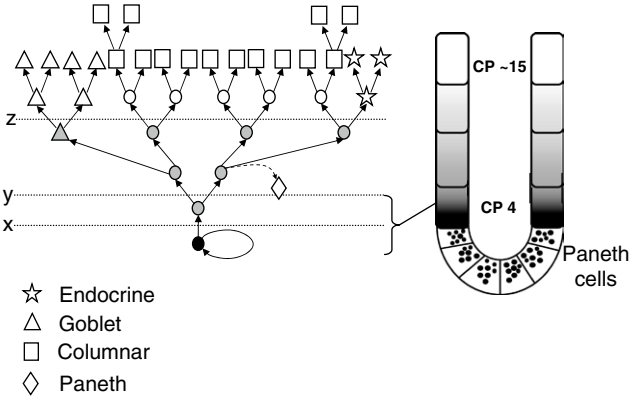


FIGURE 3-2 The cell lineage for the small intestinal crypts. It is postulated that each crypt contains four to six such lineages and, hence, four to six lineage ancestor actual stem cells, and there are about six cell generations in each lineage with at least four distinct differentiated cell types being produced. The attractive feature of this cell biological model system is that the position of a cell in a lineage can be related to its topographical position in a longitudinal section through the crypt, as shown on the right.

SKIN STEM CELLS

The first suggestion that the proliferative compartment of the epidermis, the basal layer, was heterogeneous and contained only a small sub-population of stem cells came with the development of the skin macrocolony clonal regeneration assay developed by Withers. This was soon combined with other cell kinetic and tissue organization data to formulate the concept of the epidermal proliferative unit (EPU) (see Figure 3-3). This suggested that the basal layer consisted of a series of small, functionally and cell lineage-related cells, with a spatial organization that related directly to the superficial functional cells of the epidermis, the stratum corneum. The concept indicated that the epidermis should be regarded as being made up of a series of functional proliferative units. Each unit had a centrally placed self-maintaining stem cell and a short stem cell-derived cell lineage (with three generations). The differentiated cells produced at the end of the lineage migrated out of the basal layer into the suprabasal layers in an ordered fashion, where further maturation events occurred, eventually producing the thin, flattened, cornified cells at the skin surface that were stacked into columns (like a pile of plates), with cell loss occurring at a constant rate from the surface of the column (Figure 3-3).

Such an organization is clearly evident in the body skin epidermis of the mouse, its ears, and a modified version of the proliferative unit can be clearly identified in the dorsal surface of the tongue. There has been, and continues to be, some debate as to whether this concept applies to human epidermis. In many sites of the human body a similar columnar organization can be seen in the superficial corneal layers of the epidermis. What is more difficult in humans is to relate this superficial structure to a spatial organization in the basal layer. However, the spatial organization seen in the superficial layers must have an organizing system at a level lower in the epidermis, and it does not seem unreasonable to assume that this is in the basal layer, as is the case for the mouse epidermis.

Withers developed a macroscopic, clonal regeneration assay for mouse epidermis, which generates nodules very similar in appearance to spleen colonies. Subsequently, Al-Barwari developed a microscopic clonal assay that required a shorter time interval between irradiation and tissue sampling. Both techniques are fairly labor intensive and have not been used extensively. Together, these clonal regeneration assays were interpreted to indicate that only about 10% (or less) of the basal cells have a regenerative capacity (i.e., are stem cells).

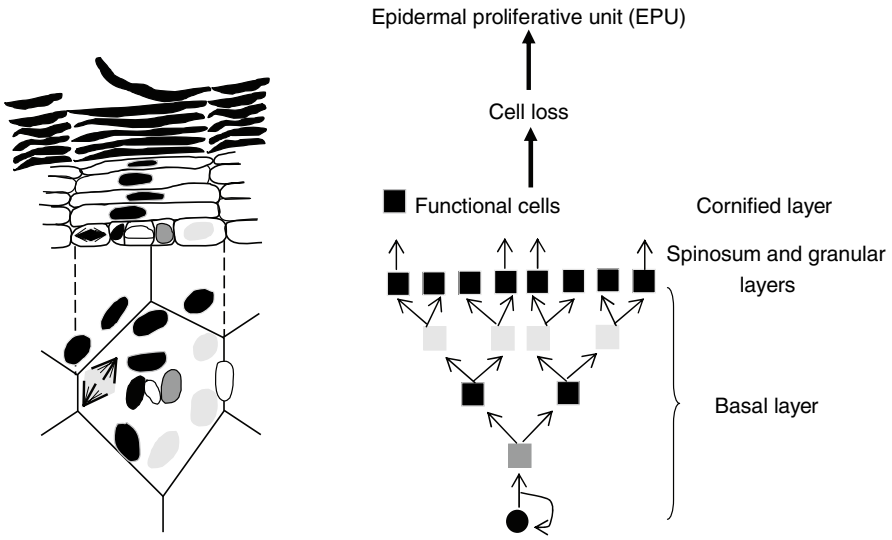


FIGURE 3-3 Diagrammatic representation of the cell lineage seen in the interfollicular epidermis and the relationship between the cell lineage and the spatial organization characterized as the epidermal proliferative unit (EPU), as seen in section view (upper portion of the figure on the left) and in surface view in epidermal sheets (lower portion of the figure on the left).

The EPU stem cells must have an asymmetric division mode under steady state cell kinetics, because there is only one such cell per EPU. The epidermal microcolony assay developed by Al-Barwari suggests that following injury, such as irradiation, surviving EPU stem cells can change their division mode from asymmetric to symmetric for a period of time to repopulate the epidermis (i.e., change their self-maintenance probability from 0.5 to a value higher than 0.5). Al-Barwari's observations also indicated that a significant contribution to re-epithelialization could come from the upper regions of the hair follicles. Studies on the structural organization of the epidermis following injury also made it clear that in order to re-establish the spatial distribution of stem cells, the epidermis undergoes a reorganization involving hyperplasia during which stem cells are redistributed and eventually establish their EPU spatial configurations.

The skin contains another important stem cell population, namely, that associated with the growing hair follicles. Hair is produced over a protracted period of time by rapid divisions in the germinal region of the growing hair follicle (termed an "anagen follicle"). This hair growth may be maintained for long periods of time—three weeks in a mouse (where the average cell cycle time may be 12 hours), months to years in humans, and more indefinite periods for some animal species such as Angora rabbits and Moreno sheep. This high level of cell division in the germinal matrix of the follicle, which has a considerable spatial polarity like the intestinal crypt, must have a fixed stem cell population residing in the lowest regions of the germinal matrix that can maintain the cell production for the required period of time. Very little is known about these stem cells. The complication with hair follicles is that in mouse and human, the growing follicles eventually contain a mature hair, and cell proliferation activity ceases. The follicle shrinks and becomes quiescent (a telogen follicle). The simplest explanation here is that the telogen follicle, which consists of far fewer cells in total than in a growing follicle, contains a few quiescent hair follicle stem cells that can be triggered back into proliferation at the onset of a new hair growth cycle. However, as discussed below, there is some controversy concerning this concept.

It is now very clear that the skin contains a third stem cell compartment, which is located in the upper outer sheath of the hair follicle below the sebaceous glands. This is sometimes identifiable by virtue of a small bulge in the outer root sheath, and so this population of cells has been referred to as the bulge cells. A whole series of extremely elegant, but complicated, experiments have shown that these bulge cells possess the ability, under specialized conditions, to reform the hair follicle if it is damaged and also to contribute to the re-epithelialization of the epidermis. It is cells from this region of the follicle that were probably responsible for the epidermal re-epithelialization from follicles seen by Al-Barwari. Cells from the bulge can make follicles during development of the skin and also re-establish the follicles if they are injured.

The controversy concerns the issue of whether bulge stem cells, which are predominantly quiescent cells, ever contribute to the re-establishment of an anagen follicle under normal undamaged situations. The simplest interpretation is that these cells are not required for this process, since in order for this to happen some very complex cell division and cell migratory pathways have to be inferred. This goes somewhat against the concept of stem cells being fixed or anchored, and also against the concept of keratinizing epithelia being a tightly-bound strong and impervious barrier. What seems likely for the skin is that the EPU stem cell and the hair follicle stem cell have a common origin during the development of the skin from the bulge stem cells, which then become quiescent and are present as a versatile reserve stem cell population that

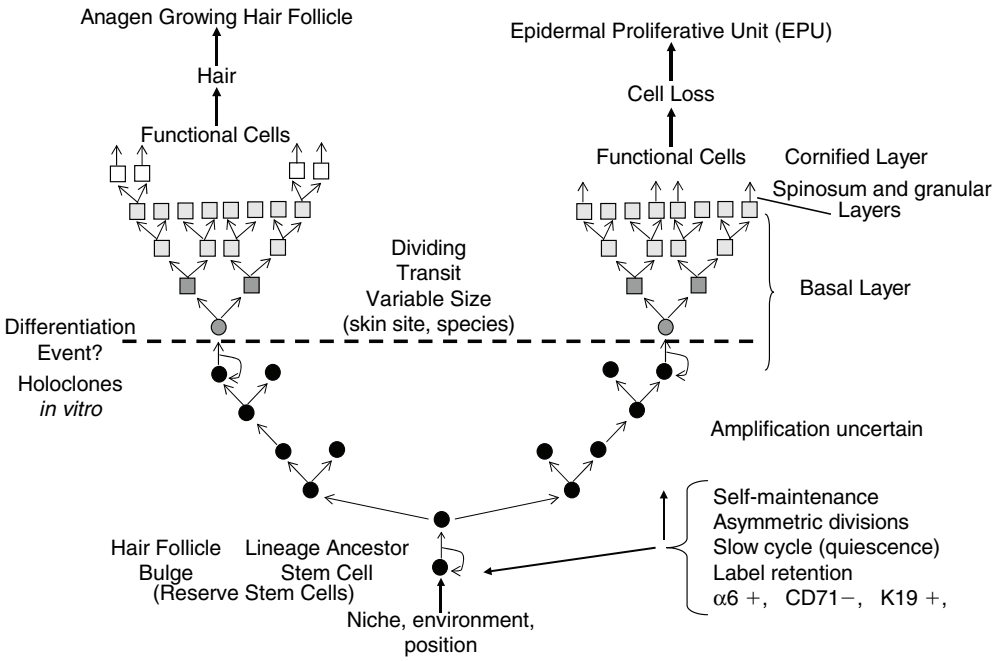


FIGURE 3-4 The complexity of the stem cell populations in mammalian skin as characterized in the mouse. A distinct cell lineage is proposed (A) for the interfollicular epidermis (EPU); (B) another for the matrix region of the growing hair follicle (anagen follicle); and (C) a potent reserve regenerative stem cell compartment which resides in the upper/outer root sheath or bulge region of the hair follicle. The stem cells in the bulge region can regenerate the epidermis, the hair follicle, and probably other structures such as the sebaceous glands.

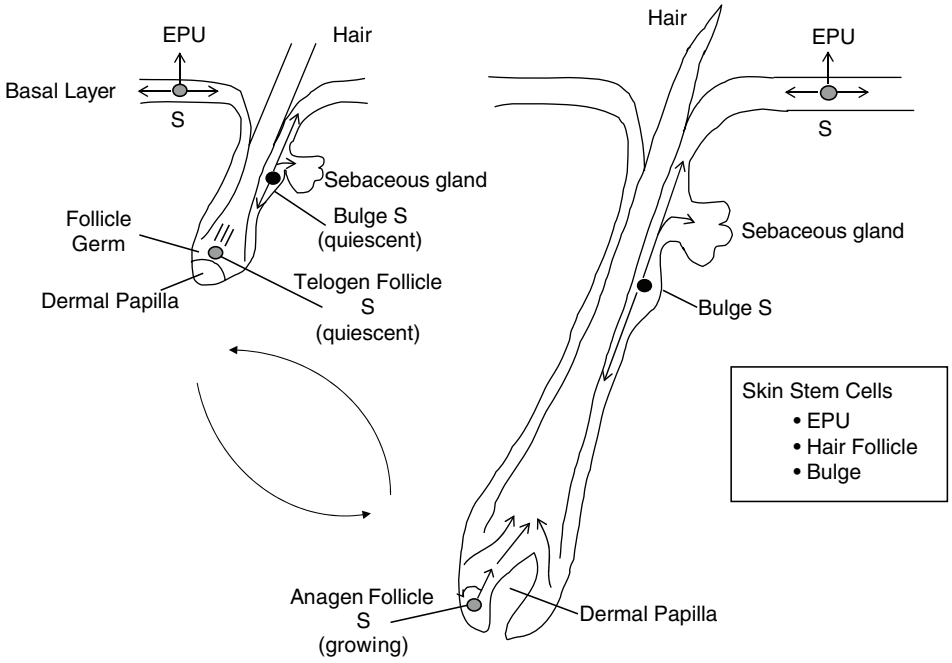


FIGURE 3-5 Diagrammatic representation of a growing anagen hair follicle and a resting or quiescent telogen follicle. The diagram shows the spatial distribution for the stem cell compartments shown in Figure 3-4.

can be called into action if the skin is injured and requires re-epithelialization (see Figures 3-4 and 3-5).

THE INTESTINAL STEM CELL SYSTEM

The intestinal epithelium, like all epithelia, is highly polarized and divided into discrete units of proliferation and differentiation. In the small intestine, the differentiated units are the finger-like villi protruding into the lumen of the intestine. These structures are covered by a simple columnar epithelium consisting of several thousand cells, which perform their specific function, become worn out, and are shed predominantly from the tip of the villus. There is no proliferation anywhere on the villus. The cell loss from the villus tip is precisely balanced in steady state

by cell proliferation in units of proliferation at the base of the villi called crypts.

Each villus is served by about six crypts, and each crypt can produce cells that migrate onto more than one villus. The crypts in the mouse contain about 250 cells in total, 150 of which are proliferating rapidly and have an average cell cycle time of 12 hours. The cells move from the mouth of the crypt at a velocity of about one cell diameter per hour, and all this movement can be traced in the small intestine, back to a cell position about four cell diameters from the base of the crypt. The very base of the crypt, in mice and humans, is occupied by a small population of functional differentiated cells, called Paneth cells. Cell migration tracking and innumerable cell kinetic experiments suggest that the stem cells that represent the origin of all this cell movement are located at the fourth position from the base of the crypt in the small intestine, and right at the base of the crypt in some regions of the large bowel.

The crypt is a flask-shaped structure with about 16 cells in the circumferential dimensions. Mathematical modeling suggests that each crypt contains about five cell lineages and, hence, five cell lineage ancestor stem cells. Under steady state kinetics, these cells are responsible for all the cell production, producing daughters that enter a dividing transit lineage of between six and eight generations in the small and large bowel, respectively (see Figures 3-1 and 3-2). The stem cells in the small intestine divide with a cycle time of approximately 24 hours and, hence, in the lifetime of a laboratory mouse divide about 1000 times. It is assumed that these cells are anchored or fixed in a microenvironmental niche that helps determine their function and behavior. The uniquely attractive feature of this model system, from a cell biological point of view, is that in the absence of stem cell specific markers, the behavior and characteristics and response to treatment of these crucial lineage ancestor cells can be studied by studying the behavior of cells at the fourth position from the bottom of the crypt in the small intestine. When this is done, one of the features that seem to characterize a small population of cells at this position (about five cells) is that they express an exquisite sensitivity to genotoxic damage, such as are delivered by small doses of radiation. They appear to tolerate no DNA damage and activate a p53-dependent altruistic suicide (apoptosis). It is believed that this is part of the genome protection mechanisms that operate in the small intestine and account for the very low incidence of cancer in this large mass of rapidly proliferating tissue.

Clonal regeneration techniques also developed by Withers have been used extensively. These techniques suggest the presence of a second compartment of clonogenic or potential stem cells (about 30 per crypt) that possess a higher radio-resistance and a good ability to repair DNA damage. These observations, together with others, suggest a stem cell hierarchy of the sort illustrated in Figures 3-1 and 3-2, with the commitment to differentiation that distinguishes dividing transit cells from stem cells occurring about three generations along the lineage. Virtually identical lineage structures can be inferred for the colonic crypts.

There has been an absence of stem cell-specific markers in the past, but some may now be available. Antibodies to Musashi-1, an RNA binding protein identified as playing a role in asymmetric division control in neural stem cells, appears to be expressed in very early lineage cells in the small intestine (see Figure 3-6).

Very recent studies have indicated that the ultimate stem cells in the crypt possess the ability to selectively segregate old and new strands of DNA at division and retain the old template strands in the daughter cell destined to remain a stem cell. The newly-synthesized strands which may contain any replication-induced errors are passed to the daughter cell destined to enter the dividing transit population and to be shed from the tip of the villus five to seven days after birth from division. Cairns developed this concept in 1975. This selective DNA segregation process provides a second level of genome protection for the stem cells in the small intestine, protecting them totally from the risk of replication-induced errors, thus providing further protection against carcinogenic risk and an explanation for the very low cancer incidence in this tissue (see Table 3-1). This mechanism of selective DNA segregation allows the template strands to be labeled with DNA synthesis markers at times of stem cell expansion (i.e., during late tissue development and during tissue regeneration after injury). The incorporation of label into the template strands persists (label-retaining cells), thus providing a truly specific marker for the lineage ancestor cells (see Figure 3-6). Figure 3-6 also illustrates some other ways in which intestinal stem cells may be distinguished from their rapidly dividing progeny.

STEM CELL ORGANIZATION ON THE TONGUE

Oral mucosae are keratinizing, stratified epithelia, similar to epidermis in their structural organization. The dorsal surface of the tongue is composed of many small, filiform papillae that have a very uniform shape and size. Detailed histological investigations, together with cell kinetic studies performed by Hume, showed that each papilla is composed of four columns of cells, two dominant and two buttressing columns. The dominant anterior and posterior columns represent modified versions of the epidermal proliferative units and are called tongue proliferative units. The cell migratory pathways were mapped (like the studies in the intestinal crypts), which enabled the position in the tissue from which all migration originated to be identified, this being the presumed location of the stem cell compartment. The lineage characterizing this epithelium is similar to that seen in the dorsal epidermis of the mouse—that

is, self-replacing asymmetrically dividing stem cells, occurring at a specific position in the tissue, and producing a cell lineage that has approximately three generations (Figure 3-7). The stem cells here have a particularly pronounced circadian rhythm.

GENERALIZED SCHEME

For the major replacing tissues of the body, hierarchical or cell lineage schemes appear to explain the cell replacement processes. These schemes may involve isolated, single

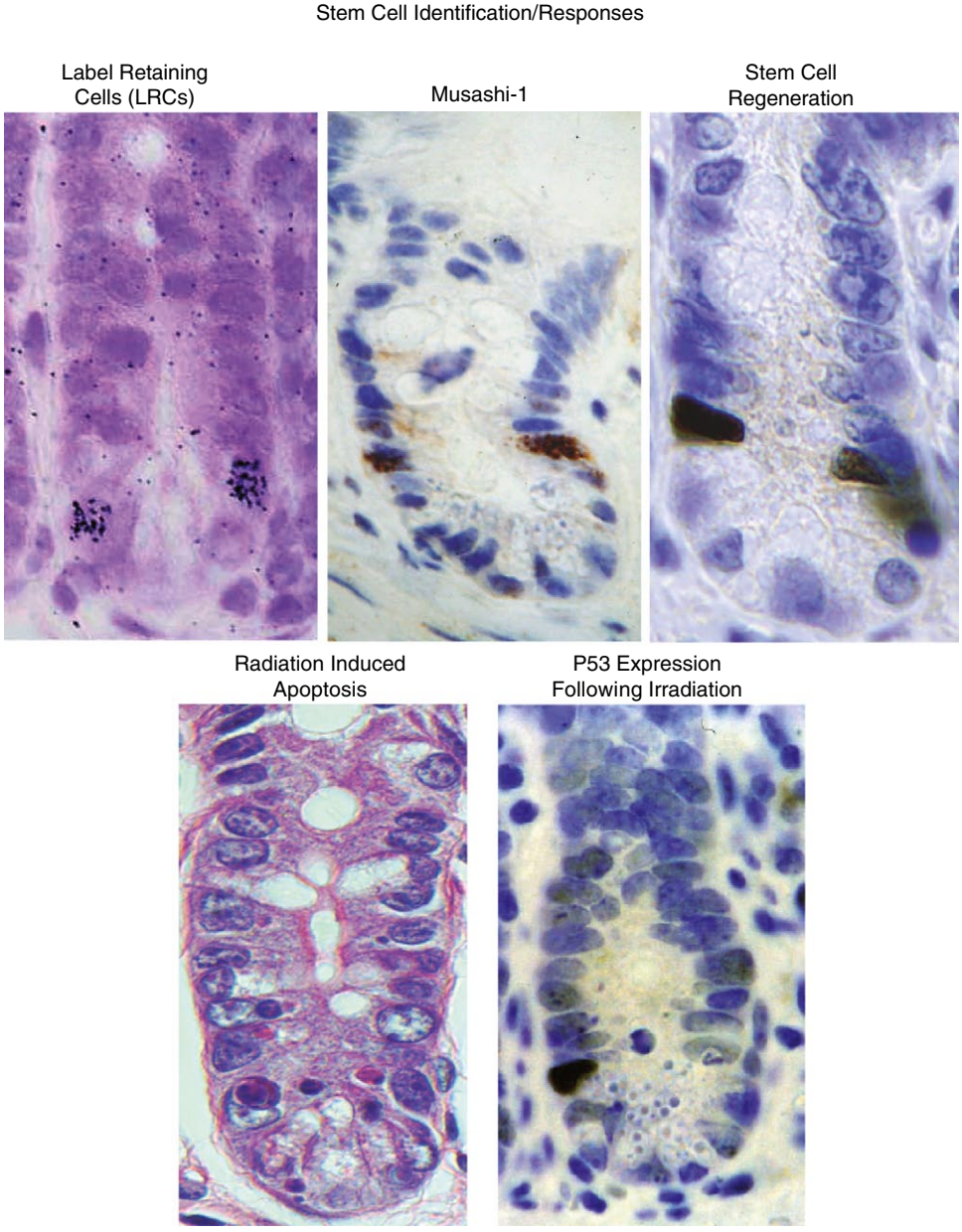


FIGURE 3-6 Photomicrographs of longitudinal sections of the small intestinal crypts from the mouse illustrating a range of possible ways of identifying the stem cell compartment. Making use of the selective strand, segregation hypothesis template strands of DNA can be labeled, generating label-retaining cells at the fourth position from the bottom of crypts. Musashi-1, an RNA binding protein, is expressed in early lineage cells and under some labeling conditions can show specificity for individual cells at around cell position 4. Part of the regenerative or potential stem cell compartment can be seen by S-phase labeling (Bromodeoyuridine labeling) at critical phases following cytotoxic injury when these cells are called into regenerative mode. The example shown here is a labeling pattern at 24 hours after two doses of 5 Fluorouracil when the only cells in S phase are a few cells scattered around the fourth position from the base of the crypt. As part of the genome protective mechanism, it is postulated that the ultimate lineage ancestor stem cells have an exquisite sensitivity to radiation and the induction of genome damage. When this happens, the cells commit suicide via apoptosis, which can be easily recognized and occurs at about the fourth position from the base of the crypt. These cells do not express p53 protein, at least at the times studied and as detectable by immunohistochemistry. However, some cells do express p53 protein at high levels following radiation exposure, and it is postulated that these are the surviving potential stem cells in cell cycle arrest to allow for repair prior to entering rapid regenerative cell cycles. Under appropriate immunohistochemical preparative procedures, individual wild-type P53 protein expressing cells can be seen at around cell position 4.

stem cells that under steady state circumstances must be presumed to divide asymmetrically, producing a dividing transit population. The size of the dividing transit population differs dramatically from tissue to tissue, the number of generations defining the degree of amplification that the transit population provides for each stem cell division. This is related inversely to the frequency that stem cells will be found within the proliferating compartment (see Figure 3-8).

For some systems, such as the bone marrow and the intestine, the commitment to differentiation that separates the dividing transit compartment from the stem cell compartment appears to be delayed until a few generations along the lineage. This generates a stem cell hierarchy with cells of changing (decreasing) stemness or, conversely, increasing commitment, leading to the concept of committed precursor cells. In the small intestine, this delay in the commitment to differentiation to a dividing transit population provides the tissue with a reserve population of potential stem cells that can repopulate the tissue if the lineage ancestor cells are destroyed; this gives an added level of tissue protection in this extremely well-protected tissue.

With regard to the bone marrow, committed precursors or even earlier cells appear to circulate in the blood and may lodge in various tissues. Given appropriate microenvironments and local signals, some of these lodged cells may be instructed to differentiate down unusual pathways. This has prompted research into using such cells to repopulate the liver of patients with specific gene defects that result in life-threatening, hepatic metabolic deficiencies.

Although the transdifferentiation theory is attractive, recent research indicates that the apparent plasticity of stem cells may be less clear-cut. Transplantation experiments in mice with specific gene disorders suggest that transplanted bone marrow cells may "fuse" with liver cells, and hence complement any gene deficiency in the hepatocytes. These hybrid cells will be viable and undergo clonal expansion. Experimental findings do, indeed, show that cells forming functional liver tissue in the gene-deficient animals have specific genetic markers for both the donor and the host animal. Our concepts of stem cells clearly require further development and refinement.

TABLE 3-1 Why Do Small Intestinal Stem Cells Not Develop More Cancers?

When one considers that the tissue has:
- 3–4 times greater mass (length)
- 1.5 times more rapid proliferation
- 2–3 times more total stem cells
- 3–4 times more stem cell divisions in a lifetime

Compared with the large intestine:
- the small intestine has 70 times fewer cancers.

SUMMARY

Stem cell concepts have evolved dramatically over the last few years from the simple ideas in the literature in the

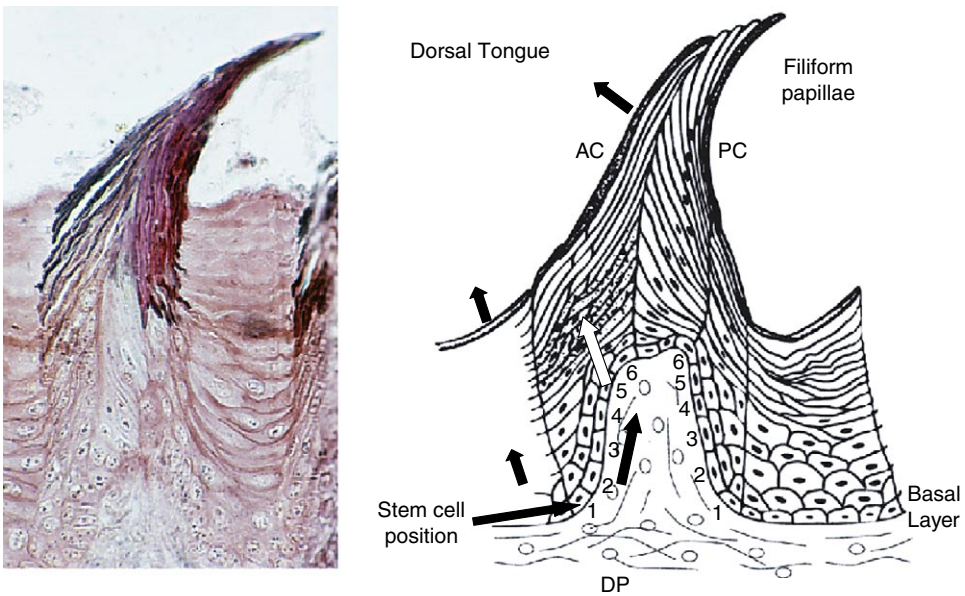


FIGURE 3-7 A histological section through the dorsal surface of the tongue (left panel) and a diagrammatic representation of this tissue showing the tongue proliferative units (the dominant anterior column AC, and posterior column PC). Cell migratory pathways have been identified based on cell positional analyses and cell marking and the location of the stem cells identified in the basal layer. The stem cells in this tissue express one of the strongest circadian rhythms in proliferation seen anywhere in the body.

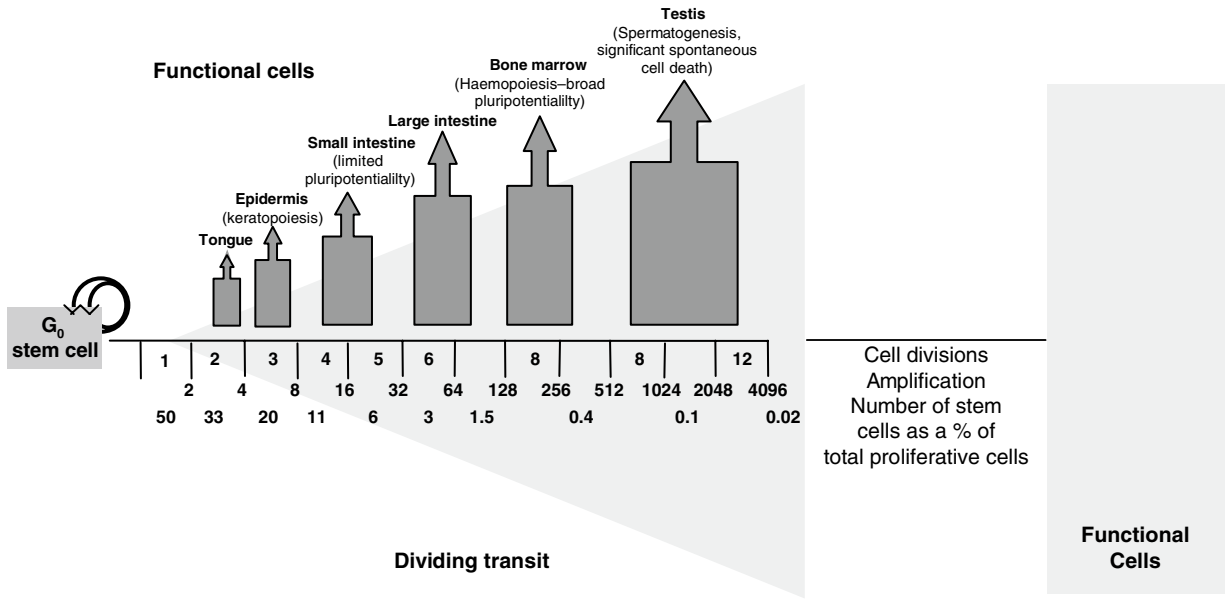


FIGURE 3-8 A diagrammatic representation of a stem cell-derived cell lineage showing the approximate positions for the number of cell generations in the dividing transit population for a range of murine tissues. Stratified keratinizing epithelia such as the tongue and epidermis tend to have the shortest lineages, and the bone marrow and the testis tend to have the longest lineages. Also shown is the degree of theoretical amplification that the dividing transit lineage provides for each stem cell division and the inverse relationship between the degree of amplification and the proportion of the proliferative compartment that the stem cells occupy.

mid-twentieth century. This has culminated in a rapid expansion of interest in both embryonic and adult tissue stem cells in the last five years with the development of interest in gene therapy and tissue engineering. This chapter explores the evolution of stem cell concepts as applied to adult epithelial tissues. These tissues are characterized by a high degree of polarization and very distinct cell maturation and migration pathways, which permit the identification of specific locations in the tissues which represent the origins of all this cell movement. Cells at the origin of the migratory pathways must represent the cells on which the tissue is ultimately dependent, and the cells that have a long-term (permanent) residence in the tissue, i.e., the stem cells. A variety of cell kinetic studies, together with lineage tracking experiments, have indicated that in the intestine, the dorsal surface of the tongue, and the interfollicular epidermis, the proliferative compartment of the tissue is divided into discrete units of proliferation, each with its own stem cell compartment. In the skin, the evolving stem cell studies suggest at least three distinct stem cell populations providing a source of cells for the epidermis, for the growing hair follicle, and a reserve regenerative highly potent population in the upper follicle region. In the small intestine there are indications that the stem cell compartment itself is hierarchical, with a commitment to differentiation occurring two to three generations down the lineage, resulting in a population of actual stem cells that perform their function in steady state, and a population of potential stem cells that can be called into action if the actual stem cells are killed. Until recently, there have been no reliable markers for adult intestinal stem cells; however, new developments have indicated ways in which these cells may be identified. Cancer is rare in the small intestinal epithelium, which is surprising since this tissue represents a large mass with many stem cells dividing many times. This suggests that effective genome protective mechanisms have evolved, and some aspects of these mechanisms have now been identified.

KEY WORDS

Actual functional stem cells The cells on which the tissue is ultimately dependent for day-to-day cell replacement.

Dividing transit cells The amplifying cells derived from stem cells that continue to divide several times before undergoing terminal differentiation (maturation) into the functional cells of the tissue.

Epidermal proliferative unit The functional group of proliferative basal cells derived from a single stem cell, together with the distally arranged functional differentiated cells.

Potential stem cell Cell that retains the capacity to function fully as a stem cell if necessary. Normally, these cells are displaced with time into the dividing transit population, but they retain the undifferentiated status of the ultimate stem cell until such time as they are displaced into the dividing transit populations.

Self-maintenance probability The probability that stem cells make other stem cells on division. It applies to

populations of stem cells rather than individual cells. In steady state it is 0.5, but during situations where stem cell populations expand it can be between 0.5 and 1.0.

Tongue proliferative unit A modified version of the epidermal proliferative unit identified in the filiform papillae on the dorsal surface of the tongue.

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Chapter 4

Direct Reprogramming of Somatic Cells to a Pluripotent State

Dirk Hockemeyer, Frank Soldner, and Rudolf Jaenisch

INTRODUCTION

Somatic cell reprogramming is the process of converting the epigenetic state of a differentiated somatic cell into a pluripotent state capable of giving rise to any cell type. Due to this ability to differentiate into all somatic cell types, pluripotent stem cells bear promise to advance biomedical research and regenerative medicine. Transplantation of specific functional cell types to replenish lost tissue has been proposed as a strategy to treat degenerative diseases such as Parkinson's disease, diabetes, and heart disease. For such therapies, the use of patient-derived cells generated by reprogramming is advantageous over conventional embryonic stem (ES) cells, as autologous cells are predicted to evade immune rejection upon transplantation.

One of the strategies proposed to generate patient-derived pluripotent cells is the transfer of a somatic nucleus into a human donor oocyte. This procedure, termed somatic cell nuclear transfer (SCNT), was successfully used to generate live cloned animals from terminally differentiated somatic nuclei. These experiments showed for the first time that the epigenetic state of a somatic cell could be reset to an embryonic state that supported the development of a new organism. Further experiments in the mouse system demonstrated the therapeutic potential of embryonic stem cell technology. In these experiments mouse ES cells were derived by SCNT in which a pathologic gene mutation was repaired by homologous recombination. When these ES cells were used as a source for transplantation therapy, organ function and viability was restored, thereby providing proof of principle for the feasibility of embryonic stem cell therapy. However, several problems have impeded the use of SCNT as a general tool to derive human ES cells for stem cell therapy: the current inability to perform SCNT with human cells; lack of knowledge of the underlying principles of this procedure; and important ethical concerns.

In 2006, a ground-breaking study by Yamanaka and colleges described a method, independent of SCNT, to generate cells with embryonic stem cell properties, rejuvenating hope for the application of pluripotent stem cells in transplantation therapies. In this seminal study it was shown that the simultaneous retroviral expression of the four transcription factors Oct4, Sox2, Klf4, and C-myc, could directly reprogram mouse fibroblasts to pluripotent ES cell-like cells. Cells derived by direct reprogramming, termed induced pluripotent stem cells (iPSCs), share all the key characteristics of ES cells. By now several groups have successfully generated iPSCs and have defined the features and essential quality criteria of iPSCs. IPSCs are morphologically indistinguishable to their ES cell counterparts and express pluripotency-related cell surface markers. IPSCs also express ES cell-specific transcription factors such as Nanog, and reactivate endogenous Oct4 and Sox2 to levels comparable to those found in ES cells. This observation, together with the finding that iPSCs show an ES cell characteristic hypomethylation of the endogenous Oct4 and Nanog promoters, suggests that iPSCs reactivate the auto-regulatory pluripotency-related transcription network. This auto-regulatory loop has been proposed to be the key mechanism for self-maintenance of an ES cell state. Furthermore, chromatin immunoprecipitation, as well as methylation analysis, revealed that the epigenetic state of iPSCs is similar to that of ES cells. The similarity of iPSCs and ES cells is further illustrated by the observation that bivalent domains characteristic for ES cells can also be identified in iPSCs. Moreover, iPSCs, like ES cells, are competent to differentiate into all three developmental germ layers when such differentiation is induced using tissue culture protocols or in teratoma formation assays after subcutaneous injection into immunocompromised mice. Importantly, it has been shown that mouse iPSCs are also capable of contributing to the germline of chimeric animals that can give rise to live offspring.

Since the first derivation of iPSCs, experimental efforts have focused on developing better tools to investigate and streamline the reprogramming process. These include optimizing the delivery of the reprogramming factors into

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somatic cells, improving reprogramming efficiency, determining the cell types amenable to direct reprogramming, and identifying potential intermediate cell states during the reprogramming process. By now iPSCs have been derived from a variety of somatic cells, including fibroblasts, keratinocytes, hepatocytes, neural stem cells, and gastric epithelial cells. Importantly, it has been shown that iPSCs can be derived from terminally differentiated cells. This was achieved by direct reprogramming of B-lymphocytes using VDJ-recombination as a genetic marker for terminal differentiation. Interestingly, cells of different somatic origin have been reported to reprogram with different kinetics and efficiencies. For example, about 0.1% of fibroblasts transduced with the reprogramming factors became pluripotent iPSCs within about three to four weeks. In contrast, keratinocytes have been reported to reprogram with efficiencies of up to 1% within two weeks after viral infection. The fact that only a small fraction of cells infected with the reprogramming factors form iPSC colonies indicates that direct reprogramming is a stochastic process. The stochastic nature of reprogramming has been proposed to be partially due the heterogeneity of the viral integrations of the reprogramming factors leading to variegation effects and differential factor expression due to viral silencing. This hypothesis is supported by experiments in which the reprogramming factors were expressed using doxycycline-inducible lentiviruses to generate iPSCs. Fibroblasts isolated from chimeric mice generated using such doxycycline-inducible iPSCs are genetically homogeneous and carry the viral reprogramming factors in copy numbers and genomic loci competent to induce reprogramming. Upon Dox administration, such "secondary" fibroblasts gave rise to "secondary" iPSCs with efficiencies greatly exceeding those found after conventional primary viral infections.

In addition to experiments studying the mechanisms of direct reprogramming, major efforts have been made to bring iPSC technology towards a therapeutic application. The first steps were taken by developing protocols that allowed the derivation of iPSCs from human cells. Two independent protocols were established using different sets of virally-delivered transcription factors to generate human iPSCs. The first protocol utilizes the same four factors (Oct4, Sox2, Klf4, and C-Myc) that were employed to isolate mouse iPSCs, the second protocol was based on the overexpression of Lin28 and Nanog in addition to Oct4 and Sox2. In both cases, pluripotent cells with characteristics of human ES cells were derived that could differentiate into cells originating from all three developmental germ layers. Subsequently, these protocols have been utilized to generate iPSCs from fibroblasts derived from patients with defined genetic disease. By now, several iPSC lines have been derived from patients with either monogenic or multigenic diseases, including Parkinson's disease, diabetes, amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA). For ALS and SMA, patient-derived iPSCs have been successfully differentiated *in vitro* into the cells affected or depleted in the respective disease. Derivation of such patient-specific iPSCs not only represents significant progress towards the use of iPSC technology in transplantation therapies, but also aids in the development of *in vitro* models for human diseases. The generation of better *in vitro* models based on the differentiation of iPSCs is especially important for human diseases such as Parkinson's disease, for which mouse models fail to fully recapitulate the pathology, and for which primary tissue is not available. Cellular *in vitro* systems based on the differentiation of patient-derived iPSCs will be helpful in identifying the pathological changes underlying complex genetic diseases. Furthermore, the ability to generate large numbers of patient-specific cells will likely facilitate the development of high-throughput screens for potential therapeutic drugs.

While the most exciting prospect for the use of iPSC technology is its application in regenerative transplantation medicine, current methods for reprogramming human cells preclude its therapeutic utility, since they rely on delivery of the reprogramming factors by retroviral transduction. Viral delivery of the reprogramming factors carries the risk of tumor formation either by gene disruption, virus mediated oncogene activation, or re-expression of the integrated transgenes. In fact, it has been demonstrated in the mouse system that iPSC-derived chimeras and progeny mice frequently develop tumors resulting from reactivation of the oncogene c-Myc. Although reprogramming has been achieved in the absence of c-Myc, although with longer latency and substantially reduced efficiency, the remaining integrated reprogramming factors could also cause tumor formation. In addition to the oncogenic potential, it should also be considered that residual transgene expression, which has been reported for most iPSCs, might influence overall gene expression in iPSCs, thereby rendering iPSCs different from human ES cells and potentially affecting their differentiation potential.

There are currently three different approaches under intense investigation to resolve these issues:

1. Reprogramming through transient expression of the reprogramming factors using either adenoviral or transient transfection strategies. Recently, reprogramming of mouse somatic cells was achieved by ectopically expressing the factors without stable integration, either by transient transfection or by adenoviral gene delivery. However, because of the substantially lower efficiency, it is unclear if similar approaches will be successful in the human system.
2. Replacement of the reprogramming factors by small molecules or protein delivery. Several screens to replace the reprogramming factors in mouse and human cells have been carried out, which lead to the replacement of a single factor. The current hypothesis about the molecular mode of action of these small

molecules is that they modulate the epigenetic state of the somatic cell, rendering the genome more accessible for the reprogramming process. However, it is not yet clear if these strategies will result in the ability to replace all of the reprogramming factors, due to the absence of the instructive effect of pluripotency-related transcription factors.

3. Genome targeting of the reprogramming factors to a single genomic locus. An alternative approach to deliver the reprogramming factors could be the targeting of a genomic safe harbor locus with an expression cassette containing all the reprogramming factors. Using such an expression cassette flanked by loxP recombination recognition sites would allow the removal of the integrated transgene by cre-recombinase expression after the derivation of the iPSCs. The only genetic modification of such iPSCs would be a single loxP site residing in a known dispensable genetic locus.

Considering the current progress in the reprogramming field, it seems conceivable that some of these approaches will eventually lead to the generation of human iPSCs without integrated reprogramming factors.

KEY WORDS

ES cells (embryonic stem cells) Stem cells derived from the preimplantation embryo with an unrestricted capacity for self-renewal that have the potential to form all types of adult cells including germ cells.

iPS cells (induced pluripotent stem cells) Cells derived by somatic cell reprogramming to a pluripotent state using reprogramming factors.

OCT4, SOX2, KLF4, c-MYC The four transcription factors first described to be sufficient to reprogram somatic cell by ectopic expression.

Somatic cell reprogramming The process of converting a differentiated somatic cell into a pluripotent stem cell like state.

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Chapter 5

Clinical Translation of Stem Cells

Olle Lindvall

INTRODUCTION

One of the most fascinating aspects of research in stem cell biology is the possibility that it could lead to the development of completely novel and radical therapies for human disorders which today cannot be effectively treated, e.g., those affecting the brain and spinal cord. All fields of stem cell research are now advancing rapidly, and both mobilization of endogenous stem cells and transplantation of stem cells or their derivatives could be considered in the clinical setting. It should be pointed out, though, that so far, stem cell therapy has been established for only very few human conditions, such as hematopoietic stem cell transplants for leukemia and epithelial stem cell-based treatments for burns and corneal disorders. All other clinical applications are experimental. Unfortunately, the great hopes with stem cell research have led to the emergence of clinics offering "effective stem cell therapies" for seriously ill patients, charging large sums of money, but without preclinical data demonstrating efficacy and assessing risks, and without transparency, rigorous oversight, and patient protection. In a recent study, Lau *et al.* (2008) analyzed whether the claims made on websites offering so-called "stem cell therapies" were substantiated in the medical literature. The vast majority of these sites over-promised the results and gravely underestimated the potential risks.

For the successful development of stem cell therapies for patients with specific diseases, clinical roadmaps should be defined, i.e., the major milestones in basic and clinical research that need to be reached and the ethical, regulatory, societal, and economic issues that need to be addressed in order to move the stem cell-based approaches to clinical application. Realistic milestones towards the clinic for stem cell therapies should be defined through close interaction between basic scientists and clinicians already at an early stage. It is necessary that the peer review process for clinical stem cell research is performed with the highest possible rigor and integrity. The responsible translation of stem cells to the clinic is crucial for success in patients for several reasons:

1. to avoid risks for major adverse effects;
2. to optimize the chances for beneficial effects;
3. to avoid major delays in the development of clinically useful therapies.

Here I will briefly discuss the different steps that should be taken towards application of stem cells and their derivatives in patients. More information as well as direct recommendations can be found on the website of the International Society for Stem Cell Research (ISSCR) (www.ISSCR.org). The ISSCR has, with the help of an international task force of experts from 13 countries, developed guidelines for the clinical translation of stem cells (Hyun *et al.*, 2008). These guidelines, which form the basis of this chapter, will be continuously reviewed and revised to accommodate scientific advancements and to address translational research issues.

CELL PROCESSING AND MANUFACTURING

As a first step towards successful clinical application, the stem cells and their derivatives must be processed and manufactured to promote as much as possible the quality, safety, purity, stability, and potency of the cells to be delivered to patients. Components of animal origin should, whenever possible, be replaced by human or chemically defined components in order to avoid transmission of animal pathogens and reaction to animal proteins. Validation of the stem cell-derived products is particularly demanding, because of the different types and sources of stem cells, differences in their modes of processing, and the biological variability (Ährlund-Richter *et al.*, 2008). The procedures for stem cell donation have to be standardized, and donors should be screened for infectious diseases and genetic diseases as appropriate. It is important that scientists and regulators develop common reference standards to ensure cell quality and safety of the cells, and to facilitate comparisons across studies. Systematic assessment of the integrity and potency of cell products is essential to minimize the risks for patients. Quality control should be conducted under expert and independent oversight at a level that is proportional to the degree of risk raised by the particular cell

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product and its intended use. If the cell products have been minimally manipulated, they will require less extensive characterization and control, as compared to extensively-manipulated cell products. Also of major importance is the source of the cells (autologous versus allogeneic), their potential for differentiation (unipotent versus multipotent), their intended use (for homologous versus non-homologous functions), their persistence in the patient, and the integration of cells in tissues or organs (naked cells versus, e.g., encapsulation). Regulatory guidelines for both gene therapy and cell therapy will apply to stem cells or their derivatives which have been subjected to genetic modification.

STUDIES WITH STEM CELLS IN ANIMAL MODELS

Preclinical studies in animal models resembling the human clinical condition are of major importance for moving stem cells towards application in patients. Proof-of-principle for efficacy should be obtained in these models, where it is also necessary to assess the dose–response relationship and possible adverse effects. Before clinical application, it is desirable to understand the biological mechanisms underlying the observed therapeutic effect, e.g., whether it is due to cell replacement, or a trophic-neuroprotective, immunomodulatory or other mechanism. Also, how to monitor the fate of the stem cell-derived cells and their function must be understood. Such knowledge will be essential for the further optimization of stem cell-based approaches. Rigorous assessment of the design and outcome of these preclinical studies through independent peer review is crucial. Preclinical testing in animal models is particularly important for stem cell-based approaches, because stem cells can act through multiple mechanisms leading to both beneficial and adverse effects. The need for animal models is especially strong in the case of extensive *ex vivo* manipulation of cells and/or when the cells have been derived from pluripotent stem cells. In most cases, small animal models (i.e., rodents) are sufficient, but large animal models may be more informative with respect to, e.g., disease complexity and how to scale-up the grafting procedure to humans.

It should be emphasized, though, that preclinical experiments in animal models may not fully reflect how transplanted human cells will behave in patients. The animal model may not mimic all aspects of the pathology of the human condition, leading to lack of efficacy in clinical trials of the stem cell product. Most importantly, there is currently little clinical experience with toxicity caused by delivery of stem cells or their derivatives to patients. Adverse effects which have not been anticipated may develop after grafting in humans. Preclinical studies of the toxicity of stem cell-based approaches is therefore of crucial importance. Cells grown for long periods or under stressful conditions in culture may become aneuploid or have DNA rearrangements, deletions, and other abnormalities that could predispose them to serious pathologies such as cancer. There is a particular concern for the potential tumorigenicity of human embryonic stem cells and induced pluripotent stem cells or their differentiated derivatives. Small animal models probably reveal only major toxic effects of stem cell interventions. The similarity of many crucial physiological functions between large animals and humans favor testing the toxicity of stem cell-based approaches in at least one large animal model. A clear plan to assess the risks of tumorigenicity for any stem cell-derived product should be developed, and this plan must be acceptable to an independent review body prior to approval of the cells for clinical studies. Since stem cells or their progeny may survive in the human body for many years after grafting, it is important to use long-term monitoring of animals to determine the later effects of stem cell therapies.

CLINICAL TRIALS WITH STEM CELLS IN PATIENTS

Human diseases which have been proposed as candidates for stem cell therapy differ widely in their degree of disability and the available therapeutic options. For example, for patients with Parkinson's disease, with virtually normal life expectancy, several effective drugs, primarily L-dopa, can be given during the first years and symptomatic treatment (deep-brain stimulation) in advanced stages. In contrast, for patients with Huntington's disease, which is a fatal disorder, there is currently no effective treatment. The characteristics of the various diseases must play a major role when considering proceeding to clinical application with an experimental and potentially risky stem cell-based approach. Thus, it is necessary that the stem cell-based approach, based on the findings in preclinical animal models, has the potential to become clinically competitive as compared to existing therapies. If an efficacious therapy already exists, the risk associated with a stem cell-based approach must be low and this approach must offer a potential advantage (e.g., better functional outcome; single procedure versus life-long drug therapy with associated side effects; reduction in long-term cost). If an efficacious therapy is not available, then the severity of the disease, especially if it is severely disabling and life-threatening, might justify the risks of a stem cell-based experimental intervention in patients.

Clinical investigations using stem cell-based products raise unique and novel issues that require specialized scientific expertise and rigorous scientific review. Therefore, the ISSCR Guidelines recommend that committees responsible for oversight of research on human subjects include stem cell-specific scientific and ethical expertise during the review of stem cell-based clinical protocols involving: (1)

products from human embryonic or other pluripotent stem cells; (2) novel applications of fetal or somatic (adult) stem cells; (3) hematopoietic or other stem cells used for applications outside established standards of care. The stem cell-specific expertise should assist investigators and human research review committees in assessing the scientific rationale of the clinical trial protocol, the *in vitro* and *in vivo* preclinical studies that form the basis for proceeding to the clinical study, and the risks for adverse events, primarily tumor development. It is crucial that maximum efforts are made to minimize the risks for all possible adverse events associated with stem cell-based approaches in clinical applications. However, in case adverse events occur, there must be a clear clinical plan for how to act, e.g., against stem cell-derived tumors.

Voluntary informed consent is of crucial importance when starting clinical trials with stem cell-based approaches. For a long time, new stem cell applications in patients will be highly experimental. Therefore, the consent process should give patients a realistic description of the degree of benefit that could be expected after delivery of stem cells or their derivatives, and how any improvement would compare with that induced by other available therapies. Importantly, the patients should also be informed that stem cell-based approaches may be associated with unique risks, such as tumor formation and long-term health effects. When the clinical trial is ongoing, careful patient monitoring and adverse event reporting will be necessary. Also, for the advancement of the stem cell field towards effective and safe therapies, it will be valuable for scientists/clinicians to publish both positive and negative results. Not in the least this will stimulate basic researchers to focus on scientific problems that are particularly important to solve, in order to contribute to the clinical success of stem cell-based approaches.

CONCLUSIONS

Clinical trials with stem cells are still premature for most disorders. However, the steady progress in stem cell biology supports the development of effective stem cell-based therapies to restore and preserve function in different human organs. For each disease it should now be possible to develop a roadmap which defines the necessary scientific and clinical advancements for stem cells towards clinical practice. The development of stem cell-based therapies for various human diseases will require major research efforts in basal stem cell biology, so that the mechanisms regulating the proliferation, migration, differentiation, survival and function of stem cells and their derivatives are much better understood, and can be effectively controlled. Strategies to prevent tumor formation from pluripotent stem cells must also be developed. Functional efficacy of stem cells and their mechanisms of action should be demonstrated in animal models with pathology and symptomatology resembling the human disease. Still, it may be difficult to translate data obtained in animals to humans because of species differences in degree of plasticity, and incomplete knowledge about disease mechanisms. It should be understood how to influence the pathological tissue environment including inflammatory and immune reactions to allow for efficient repair. Finally, it must be remembered that even if the biology of stem cells is exciting, the clinical usefulness of stem cell-based therapies will be determined by their ability to provide patients with safe, long-lasting, and more substantial improvements of the quality of life, as compared to other treatments.

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Part II

Basic Biology/ Mechanisms

Chapter 6

Molecular Bases of Pluripotency

Fatima Cavaleri and Hans Schöler

INTRODUCTION

Early mammalian embryogenesis is characterized by a gradual restriction in the developmental potential of the cells that constitute the embryo. The zygote and single blastomeres from a two to four cell embryo are totipotent. As the embryo continues to cleave, the blastomeres lose the potential to differentiate into all lineages. The blastocyst is the first landmark of the embryo in which lineage restriction is apparent. At this stage, the outer cells of the embryo compact into the trophectoderm, from which the placenta will derive. The inner cells, termed inner cell mass (ICM), will give rise to all cell lineages of the embryo proper, but cannot contribute to the trophoectoderm, and thus are considered pluripotent. Once isolated and cultured *in vitro* under permissive conditions, the ICM may be propagated as an embryonic stem (ES) cell line. As a matter of fact, these cells are the *in vitro* substitutes for embryos in the search for the genetic switches and molecular mechanisms required to ensure pluripotency. Mutations affecting the ability of ES cells to self-renew or differentiate and contribute to distinct cell lineages provide the necessary tools to unravel the molecular network underlying pluripotency.

In an attempt to define the molecular basis underlying pluripotency, we will focus on four main areas:

1. Influence of the IL6 family of cytokines on ES cell pluripotency and self-renewal;
2. Extrinsic determinants of pluripotency;
3. Intrinsic determinants (i.e., transcription factors) of pluripotency;
4. Epigenetic configuration of pluripotent cells.

CELLULAR MODELS OF PLURIPOTENCY

Three different types of pluripotent cell lines are currently available as cellular models for the study of mechanisms of pluripotency: EC, ES and EG.

EC

Embryonal carcinoma (EC) cells are historically the first pluripotent stem cells derived from mouse embryonic or fetal tissues. EC cells are self-renewing undifferentiated cells derived from teratocarcinomas—malignant tumors containing undifferentiated cells mixed with various differentiated tissues of the three primary germ layers. Teratocarcinomas, first observed in some strains of mice as naturally gonadal developing tumors, arise from pre-implantation/pre-gastrulating embryos or primordial germ cells (PGCs) that had been ectopically grafted *in vivo*. EC cells can re-form teratocarcinomas *in vivo* and differentiate *in vitro* when cultured in suspension. They provide a suitable model system to study cellular commitment and differentiation, but have a major disadvantage: EC cells are tumor cells and consequently they are typically aneuploid. Although they could integrate into a developing embryo and contribute to adult tissues, their contribution was extremely poor and lacked consistency and reproducibility. Most importantly, EC cell contribution to the germline was shown to be a very rare event.

ES

EC cells set the technical stage for derivation and handling of ES cells a few years later. In 1981 two different scientists, Evans and Martin, reported the establishment of pluripotent cell lines from blastocyst stage mouse embryos. The pluripotentiality of these cells was considered to be proven by their capability to form teratocarcinoma upon subcutaneous injection into syngenic mice, and to differentiate from embryoid bodies into tissues of all germ layers *in vitro*. Although prone to discard the Y chromosome, ES cells are in general euploid and constitute an ideal, direct *in vitro* link to and from the embryo. In 1984 Bradley showed that pluripotent ES cells could be efficiently used to alter the mouse body composition and germline throughout generations. A few years later, the basis of modern mouse developmental genetics was laid down with the generation of genetically modified mice derived from ES cells that had been manipulated by retroviral or homologous recombination methods.

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EG Cells

In addition to EC and ES cells, a third type of pluripotent stem cells, called embryonic germ or EG cells, has been isolated from mouse. Migratory PGCs form colonies that are morphologically indistinguishable from ES colonies when grown on feeder cells in the presence of serum and a cocktail of growth factors, namely LIF (leukemia inhibitory factor), bFGF (basic fibroblast growth factor), and SCF (stem cell factor). Like ES cells, EG cells show full developmental capacity, being able to differentiate into derivatives of all germ layers *in vitro*, form teratocarcinoma *in vivo*, and contribute to all tissues of chimeric mice, including germline, upon injection into host blastocysts.

Human ES and EG Cells

In a way similar to mouse, pluripotent embryonic stem and germ cells have been derived from human blastocysts and PGCs. They are capable of differentiating into cells of all three germ layers *in vitro* and of forming teratocarcinoma when transplanted under the skin of a recipient mouse. Unlike their murine counterpart, human ES cells can even give rise to cells of the trophoectoderm lineage when cultured in the appropriate conditions.

In summary, pluripotent murine embryo-derived ES and EG cells exhibit unique properties which make them an extremely powerful model system to unveil the molecular basis of pluripotency both *in vivo* and in *vitro*:

- Unlimited self-renewal: they can be grown in large numbers and indefinitely passaged *in vitro*;
- Stable karyotype;
- Refractoriness to senescence, i.e., they are virtually immortal;
- Highly efficient and reproducible differentiation potential: they can give rise to derivatives of all three embryonic germ layers *in vitro* and *in vivo*;
- Germline colonization;
- Clonogenicity: they grow as separate colonies that can be expanded as independent subclones following genetic manipulations;
- High versatility to genetic manipulation without loss of pluripotency, as introduction of foreign DNA does not affect their ability to be fully integrated into the founder tissue of a host embryo or to colonize its germline.

THE EMBRYONIC STEM CELL ENVIRONMENT: CYTOKINES AND PLURIPOTENCY

The Stem Cell Niche

In many cases the culture of stem cells has been complicated by the lack of knowledge of their cellular environment or niche. Hematopoietic stem cells, for example, were identified more than 40 years ago, but conditions still have to be established to ensure their maintenance *in vitro*. Additionally, they might require complicated three-dimensional arrangements of specific stromal cells in order to proliferate.

The ES Cell Niche

In contrast, ES cells are relatively easy to be handled technically. The first murine embryonic stem lines were established by culturing early embryos on a layer of mitotically inactivated mouse embryonic fibroblasts. Without such a "feeder" layer, cultured embryonic cells would not remain pluripotent, *suggesting that fibroblasts either promote self-renewal or suppress differentiation, or both*.

LIF and Other Cytokines

Embryonic fibroblasts maintain pluripotency of ES cells by secreting a factor, which was identified as leukemia-inhibiting factor, also known as differentiation inhibiting activity (DIA). LIF is a member of the interleukin-6 family of cytokines, including IL-6, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1). The IL-6 family cytokines are structurally and functionally related. They act on a variety of cells, i.e., they are pleiotropic, and can mediate proliferation or differentiation or both, according to the target cell types. For example, LIF, OSM and IL-6 are competent to induce myeloma growth and to inhibit macrophage differentiation of M1 cells. The redundancy in biological function is mainly due to the structural similarity of the receptor complex involved in signal transduction (see below).

Redundant Cytokine Functions

The absence of a developmental phenotype in IL-6-, LIF-, and CTNF-null mice confirms that IL-6-related cytokines are indeed functionally redundant. However, LIF mutant females are infertile, as the interaction between embryo and uterine wall (decidual reaction) strictly depends on a surge of estrogen on the fourth day of gestation, which coincides also with a surge in LIF production by the uterus. As a consequence, LIF−/− females fail to support embryo implantation. However LIF−/− embryos can implant and develop to term in a normal uterus.

ES self-renewal dependence on cytokine supply can be attributed to several factors. LIF may influence the rate of cell proliferation or cell cycle progression and act on the stem cell phenotype by activating a signaling cascade which operates on the up- or/and down-regulation of genes that are exclusively expressed in "pluripotent" and differentiated

cells, respectively. A complete and systematic analysis of the target genes lying downstream of the LIF-induced signaling pathways is necessary to clarify the cytokine *modus operandi* on the ES cell phenotype.

CYTOKINE-RECEPTOR BINDING ON ES CELLS: MULTIPLE RELAY STATIONS

Receptor Structure

The receptors involved in the IL-6 family cytokine signaling cascade belong to the cytokine receptor class I family. The extra-cellular domain of all members of cytokine receptor class I family is composed of a variable number of fibronectin type III modules. Two of the fibronectin modules are conserved among all members of the family and constitute the cytokine-binding module. The cytoplasmatic domain of the receptor contains three conserved motifs, called box 1, box 2, and box 3 in a membrane proximal to distal order, and lacks intrinsic kinase activity. These three subdomains are responsible for transmitting the extracellular signal into the cytoplasm.

Receptor Dimerization

Binding of the IL-6 family cytokine to their cognate receptors leads to homodimerization of gp130 or heterodimerization of gp130 with the cytokine cognate receptor. For example, IL-6/IL6R and IL-11/IL-11-R complexes induce gp130 homodimerization. Both LIF and CT-1 bind to LIFR and induce LIFR/gp130 heterodimerization. CTNF engages LIFR/gp130 as a signaling competent complex through an association with CTNF/CTNFR, whereas OSM engages the LIFR/gp130 or OSMR/gp130 by binding to the gp130 portion of the heterodimers (see Figure 6-1). All the described receptor complexes share gp130 as the common component critical for signal transduction, which explain the observed redundancy in cytokine functions.

Expression of Cytokine Receptors

Although gp130 is widely expressed in various tissues, ligand-specific receptor components display a more restricted expression. In the mouse blastocyst LIF and LIFR have a reciprocal pattern of expression: the cytokine is expressed in the trophectoderm and the receptor in the ICM.

LIFR, OSMR, and CTNFR are expressed in ES cells, consequently CT-1, OSM, CNTF, and LIF are interchangeable in supporting ES cell derivation and maintenance of ES cells in culture. IL-6 and IL-11 cannot substitute for CT-1, OSM, and CNTF as the IL-6 and IL-11 receptors are not expressed in ES cells. However, IL-6 can prevent ES differentiation if delivered in conjunction with a soluble form of the IL-6 receptor, which retains ligand binding activity and capability to induce gp130 homodimerization (see Figure 6-1).

Cytokine Receptor Function *In Vivo*

LIFR-null embryos die shortly after birth, and exhibit reduced bone mass and profound loss of motoneurons. Embryos homozygous for the gp130 mutation die between 12 and 18 days post coitum (dpc), because of placental, myocardial, hematological, and neurological disorders. CTFR deficient mice exhibit perinatal death and display profound motor neuron deficits.

Gp130 and LIFR Function in Diapause and ES Cells

The late embryonic lethality of gp130−/− fetuses is in conflict with gp130 requirement for ES cell self-renewal

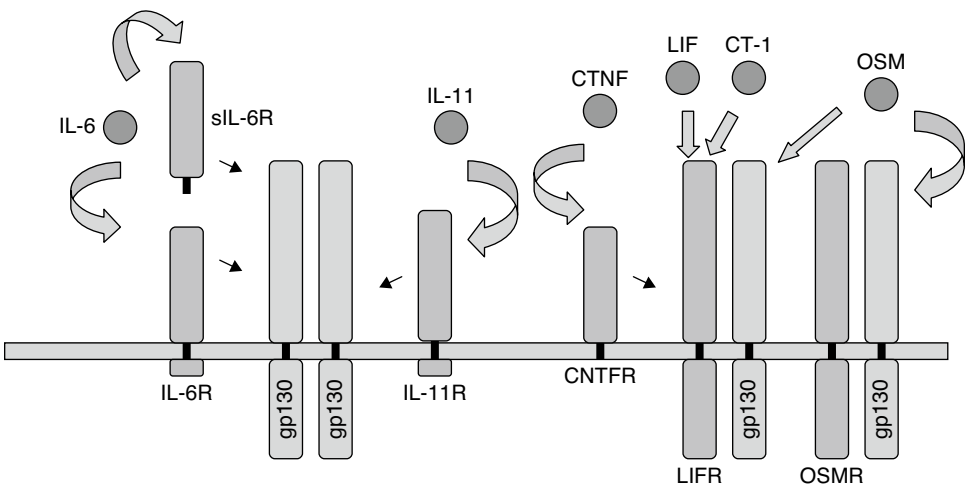


FIGURE 6-1 Schematic structure of the cytokine receptor complexes sharing gp130 as a common subunit. IL-6/IL6R and IL-11/IL-11-R complexes induce gp130 homodimerization. Both LIF and CT-1 bind to LIFR and induce LIFR/gp130 heterodimerization. CTNF engages LIFR/gp130 as a signaling competent complex through an association with CTNF/CTNFR, whereas OSM engages the LIFR/gp130 or OSMR/gp130 by binding to the gp130 portion of the heterodimers.

in vitro. Since delayed or quiescent blastocysts were used in the initial experiments of ES cell derivation, the possibility that cytokine receptors may have an embryonic function in the quiescence embryo state was investigated. Lactating females can conceive while still nursing their pups, but cannot support blastocyst implantation because they do not produce estrogen at the fourth day of gestation. Consequently, embryonic development is arrested and resumes under favorable conditions for optimal development. This phenomenon, termed diapause, can be artificially induced by ovariectomy after fertilization. The embryos reach the blastocyst stage, hatch from the zona pellucida and float *in uterus* in a quiescent status for up to four weeks. In this scenario, the epiblast, which normally preserves its pluripotent status for about three days (from 3.5 dpc, when it forms, up to 6.5 dpc, when gastrulation starts), can be maintained for longer time periods and resumes development when an estrogen-rich environment is established.

LIFR−/− and gp130−/− delayed embryos are unable to resume embryogenesis after twelve and six days of diapause, respectively. The number of cells that constitute the ICM of delayed gp130−/− blastocysts gets gradually reduced by apoptosis during a six-day period of diapause. Moreover, ICMs isolated from delayed gp130 null blastocysts cannot form a pluripotent outgrowth *in vitro*, as they differentiate exclusively into parietal endoderm.

Thus, it appears that maintenance of the epiblast during diapause is temporally dependent on different cytokines and that gp130 plays a more critical role than LIFR in this process. Two models may explain why epiblast cells enter apoptosis in the absence of gp130 signaling:

1. gp130 relays a cell survival signal from the extracellular compartment to the nucleus. This model is supported by the anti-apoptotic activity of the transcription factor STAT3 in a variety of cells.
2. gp130 suppresses epiblast differentiation. In the absence of gp130 signal, epiblast cells may differentiate inappropriately (as shown by the endoderm formation solely), and consequently die.

SIGNAL TRANSDUCTION: CASCADES TO THE STEM CELL NUCLEUS

Homo- or heterodimerization of gp130 results in the activation of receptor-associated kinases of the Janus family Jak1, Jak2 and Tyk2. These tyrosine kinases constitutively interact with the conserved regions box 1 and box 2 of gp130. The receptor complex is inactive until ligand-induced receptor dimerization brings the associated Jak kinases within sufficient proximity to allow *trans*phosphorylation and activation of the kinase catalytic domain. Activated Jaks phosphorylate specific tyrosines on the intracellular domain of gp130, creating docking sites for the recruitment of SH2 proteins to the activated receptor complex. When gp130 is phosphorylated, several signaling pathways, involving STAT1, STAT3, the SH2-domain containing tyrosine phosphatase (SHP2), ERK1/ERK2 (extra-cellular signal receptor kinases) or mitogen activated kinases (MAPK), and phosphatidylinositol-3 kinase (PI3K), are activated.

STATS: LATENT TRANSCRIPTION FACTORS TRANSMITTING SIGNALS

The STAT Family

STAT proteins belong to a group of latent cytoplasmic transcription factors that play a central role in transmitting signals from the cytoplasmic membrane to the nucleus, hence their name (signal transducers and activator of transcription). Seven major STAT proteins have thus far been identified in mouse (STAT1 to STAT6, including STAT5a and STAT5b). With the exception of STAT4, which is restricted to myeloid cells and testis, STAT factors are ubiquitously expressed. They are activated in many cell types by a broad range of cytokines, growth factors and interferons (IFNs) and they are substrates for tyrosine kinases of the Src and Jak families.

Structure

STAT proteins share several conserved structural and functional domains. A tetramerization and a leucine zipper-like domain are located at the amino terminus, followed by a DNA binding domain, a Src homology domain 3-like region (SH3, proline rich motif binding domain), a Src homology domain 2 (SH2), a critical site of tyrosine phosphorylation (Y705 in STAT3), and a carboxy-terminal transactivation domain. No evidence has emerged so far to suggest SH3 function.

The SH2 domain plays three important roles:

- Recruitment to activated receptor complexes
- Interaction with Jaks
- STAT dimerization and DNA binding.

Regulation

The regulation of STAT signaling is mostly post-translational and involves both tyrosine and serine phoshorylation. Phosphorylation of the conserved tyrosine (Y701 for STAT1, Y705 for STAT3) results in dimerization of STAT1/STAT3 through the intermolecular interaction of the SH2 domains and the domain containing the phosphorylated tyrosine. STAT1 and STAT3 homo and heterodimers translocate to the nucleus, where they activate gene transcription by binding to specific DNA sequences. Consistent with the requirement for dimerization-induced activation, the consensus binding sites are symmetrical dyad sequences.

STAT3

STAT3 was originally identified as an acute response factor which, upon IL-6-family cytokine stimulation, induces the expression of a variety of genes, referred to as acute response genes, whose expression dramatically increases with tissue injury and inflammation.

Targeted disruption of STAT3 gene *in vivo* leads to embryonic lethality. STAT3 null embryos develop into elongated egg cylinders, but degenerate at around E7.0. At this stage wild-type embryos start to express STAT3 in the visceral endoderm. Embryonic lethality is then explained as a consequence of the failure in establishing metabolic exchanges between embryo and maternal blood. Thus, STAT3 plays a crucial role during embryonic development that cannot be compensated by other members of the STAT family.

LIF and STAT3

The initial studies conducted on LIF-dependent transcriptional activation in embryonic stem cells showed induction of a DNA binding activity that correlated with LIF treatment. Steady state levels of STAT1, 3, 5, and 6 were unaffected by LIF treatment. In contrast, co-immunoprecipitation experiments indicated STAT3 as being a component of the tyrosine-phosphorylated complex formed upon LIF induction. In addition, tyrosine kinase inhibitors were shown to impair formation of the activated STAT3 complex and to alter undifferentiated ES cell morphology versus a differentiated phenotype.

STAT3, ES Cell Renewal and the Undifferentiated State

Identification of STAT3 as a key determinant of ES renewal came from the elegant studies conducted in Smith's and Yokota's laboratory at the end of the 1990s. Granulocyte colony-stimulating factor receptor (G-CSF-R) belongs to the class I cytokine receptor family and is not expressed in ES cells. In order to characterize the functional role of the gp130/LIFR intracellular domains or residues in signal transduction, chimeric receptors constituted of the extra-cellular domain of G-CSF-R fused to the transmembrane and cytoplasmic region of gp130 or LIFR were engineered. G-CSFR/gp130 and G-CSF-R receptors can support ES self-renewal in the presence of G-CSF at high and low efficiency, respectively, whereas the G-CSF-R/LIF-R chimera does not support formation of stem cell colonies. This result suggests that gp130 is an essential component in signaling self-renewal in ES cells and that G-CSF-R and gp130 intracellular domains activate common signaling pathways.

The intracellular domain of gp130 harbors four consensus motifs, **YXXQ**, whose phosphorylated tyrosines were shown to function as docking sites for STAT3. Single mutation of these tyrosine residues (Y126, 173, 265, and 275, enumeration starting from the transmembrane domain) did not affect self-renewal appreciably (see Figure 6-2). Rather, mutations of Y265/275 completely abolished STAT3-induced binding activity and formation of undifferentiated colonies. These data demonstrate that STAT3 docking sites are essential in mediating transmission of the signal from gp130 to STAT3 in self-renewing ES cells, although the tyrosine residues are not functionally equivalent. Moreover, conditional expression of STAT3F, containing a tyrosine 705 into phenylalanine substitution, caused complete differentiation of ES cells even in the presence of LIF.

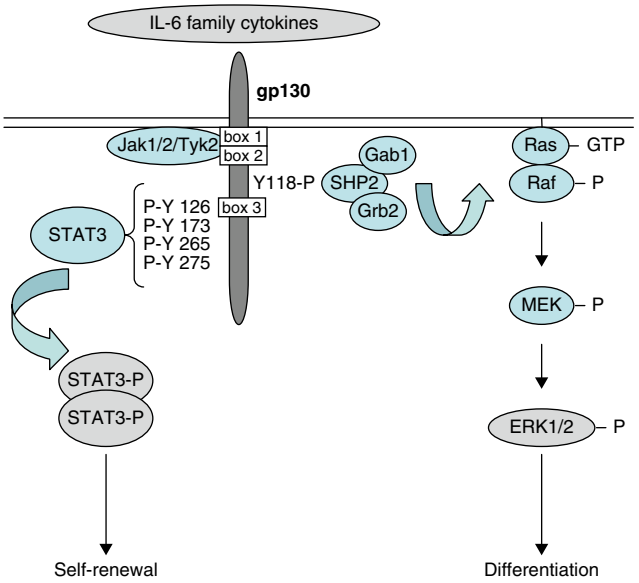


FIGURE 6-2 Schematic description of the signaling pathways induced by the IL-6 family cytokines. Following gp130 hetero- or homodimerization, activated JAKs phosphorylate the intracellular domain of gp130 on Y126, 173, 265, 275, and 118. STAT3 association with gp-130 phosphorylated Y126-275 leads to STAT3 phosphorylation, dimerization and translocation to the stem cell nucleus. Association of SHP-2 with phosphorylated gp-130 Y118 leads via adaptor proteins to activation of the Ras pathway and translocation of ERK1/2 to nucleus. STAT3 activation induces ES self-renewal, whereas ERK activation causes cell differentiation.

In order to uncouple STAT activation from any signaling pathway induced at a membrane receptor by extra-cellular factors, Matsuda and collegues constructed a fusion protein composed of the STAT3 entire coding region and the ligand-binding domain of the estrogen receptor. The STAT3ER chimeric protein specifically translocates into the nucleus in the presence of the synthetic steroid ligand, 4-hydroxytamoxifen (4OHT). Parental ES and ES expressing STAT3ER were grown in the presence of LIF or 4OHT. The compact colonies formed by STAT3ER ES in the presence of either LIF or 4HT clearly demonstrated that STAT3 activation is sufficient to maintain the undifferentiated phenotype of

ES cells. Most importantly, besides being morphologically undifferentiated, cells grown for one month in 4OHT widely contributed to all tissues of chimeric mice.

STAT3 Independent ES Cell Renewal

The striking paradox created by STAT3- or gp130-null embryo phenotypes and the strict dependence on gp130-STAT3 signaling pathways for maintenance of pluripotent ES cells is suggestive of the existence of alternative pathways governing pluripotency *in vivo*. Dani and colleagues presented some evidence of STAT3-independent signaling pathways operating in self-renewing ES cells. When ES cells are grown at high density in the absence of LIF, the newly differentiated cells start synthesizing LIF, which in turn allows the expansion of undifferentiated cells. In order to eliminate the LIF-dependent pathways of self-renewal, a LIF deficient ES cell line was generated. Strikingly, LIF null ES cells can still produce undifferentiated colonies, though at lower efficiency than wild type or heterozygous lines when induced to differentiate. A differentiated, LIF-deficient, parietal endoderm-like cell line was derived and shown to support ES cell propagation via production of a soluble activity named ES cell renewal factor (ESRF). ES cells grown in the presence of ESRF retain their pluripotent character, as they can still contribute to any tissues upon blastocyst injection. ESRF does not operate via LIFR or gp130, as it is effective on LIFR deficient ES cells and is not blocked by anti-gp130 antibodies. Additionally, STAT3 is not induced in the presence of ESRF.

SHP-2/ERK SIGNALING

STAT 1 and 3 are only two of the downstream effector molecules induced by cytokine signaling via gp130. LIF treatment of ES cells increases MAP kinase activity and induces phosphorylation of ERK1 and ERK2. The bridging factor between cytokine receptor and MAP kinase is the widely expressed tyrosine phosphatase SHP-2.

SHP-2 Structure

SHP-2 contains two N-terminal SH2 domains and a C-terminal catalytic domain. SHP-2 interacts with the intracellular domain of Gp130 through phosphorylated tyrosine 118, located inside the consensus **YSTV** sequence. Recruitment to the activated receptor induces SHP-2 phosphorylation, which leads to an increase in phosphatase activity by preventing an intramolecular interaction between the SH2 and the catalytic domain of SHP2. In BAF-B03 cells, mutation of gp130 tyrosine 118 into phenylalanine was shown to block SHP-2 phosphorylation and induction of ERK2 activation.

SHP-2-ERK Signaling and ES Cells

G-CSF responsive transgenic ES cells provided an excellent tool for establishing the role played by the SHP-2-ERK signaling in ES cell propagation.

A mutant G-CSFR-gp130 chimeric receptor that cannot be phoshorylated on Y118 (and that consequently cannot induce SHP-2 phosphorylation) does not impair the self-renewal of ES cells (see Figure 6-2). In contrast, the mutated chimera makes ES more sensitive to G-CSF, as they can be maintained at a lower concentration of G-CSF (1000-fold less) than the one required by ES cells expressing the unmodified receptor. Moreover, the signal started at the phosphorylated Y118 mediates attenuation of activated STAT3, as cells expressing the G-CSFR-gp-130 mutant chimeric receptor show a slower decay of phosphorylated STAT3.

Enhanced ES self-renewal is observed when either a catalytically inactive SHP-2 is overexpressed or ERK phosphorylation is chemically blocked. These results indicate that SHP-2/ERK activation is not required for the maintenance of self-renewal signaling, they rather inhibit it. This conclusion is confirmed by enhanced LIF sensitivity and increased proliferation rates observed in ES cells, and embryoid bodies derived thereof, expressing a ΔSH2-SHP-2 protein. However, cardiac/epithelial differentiation of SHP-2 mutant cells is inhibited and delayed, indicating that SHP-2 plays a positive role in ES differentiation. In conclusion, ES cell self-renewal is a consequence of the precise balance of antagonistic signaling pathways.

SHP-2 Communicates With ERKs

Activated ERKs undergo nuclear translocation, which enables them to modulate the activities of transcription factors that govern proliferation, differentiation, and cell survival. SHP-2 interaction with two adaptor molecules, Grb2 and Gab1, provides two alternative routes that couple cytokines to the activation of ERK pathways. SHP-2/Grb2 association leads to Ras activation through the GTP-GDP exchange protein SOS. SHP-2 has also been shown to associate with the scaffold Grb2-associated binder protein Gab1 and PI3-kinase and activate ERK MAP kinases via a Ras dependent pathway.

Grb2 and Gab1 Adaptor Proteins

Like most adaptor proteins, Grb-2 contains SH2 and SH3 domains. The SH2 domain mediates Grb2 binding to SHP-2 while the SH3 domain mediates interaction with SOS. Tyrosine phosphorylation of Gab1 following stimulation with cytokines depends on the gp130 site (Y118) docking SHP-2, but is independent from the gp130 C-terminal domain interacting with STAT3. Gp130-mediated ERK2

activation is enhanced by Gab1 expression and inhibited by a dominant negative Ras.

Functional Ablation of Grb2 in Embryos and ES Cells

Null mutation of the Grb2 gene leads to embryonic lethality at around 7.5 dpc. The differentiation potential of Grb2-null ICM cells is compromised as cultured blastocysts lack visceral and parietal endodermal cells. The ability of Grb2 to support endoderm differentiation is abrogated by mutation in either the SH2 or SH3 domain. Interestingly, transformation of Grb2-deficient ES cells with an activated variant of Ras restores endoderm differentiation, indicating that the Grb2-Ras-MAPK pathway is essential for early specification of the endoderm tissues.

FGF4 AND ERK

Fibroblast growth factor 4 (FGF4) is produced by ICM cells, and was initially postulated to function in an autocrine fashion to promote the proliferation and expansion of the ICM. This hypothesis derived from two different observations:

1. FGF4- and fibroblast growth factor receptor 2(FGFR2)-null embryos cannot form an egg cylinder and die soon after implantation.
2. When cultured *in vivo*, both FGF4- and FGFR2-null ICMs fail to expand and eventually degenerate.

In contrast to the embryo ICM or epiblast, FGF4−/− ES cells are able to proliferate in the absence of the growth factor, suggesting that *in vitro* FGF4 is not required for ES cell proliferation.

ES stimulation with FGF4 results in massive increase in Erk1/2 phosphorylation, whereas ES culture in the presence the FGFR receptor inhibitor SU5402 elicits a strong decrease in phospho-Erk1/2 levels, indicating that FGF4 constitutes the major stimulus for the activation of Erk signaling in mouse ES cells.

Since FGF4 is produced in an autocrine fashion by undifferentiated ES cells in order to establish the role of FGF4 autocrine production on ES self-renewal and commitment, FGF4 signaling was abrogated by treatment with chemical inhibitors of the FGF receptor or by genetic ablation of either FGF4 or Erk2. In all three cases, interference with the FGF signaling did not affect propagation of undifferentiated ES cells. However, Fgf4- and Erk2-null ES cells are unable to differentiate along the neural or mesodermal lineage, and display sustained expression of *Oct4* and *Nanog*. Addition of FGF4 to FGF4-null ES cells abrogates the blockade in neural/mesoderm differentiation, indicating that FGF/Erk stimulation is directly responsible for forcing ES cells to exit self-renewal and for inducing neural/mesoderm lineage commitment.

WNT SIGNALING

The canonical Wnt pathway is initiated upon Wnt protein binding to the Frizzled receptor on the cell membrane. Activation of the pathway leads to inhibition of Glycogen Synthase Kinase 3 (GSK3), which functions in inducing the proteasome-mediated degradation of cytoplasmic β-catenin. Thus, GSK3 inhibition results in increased nuclear accumulation of β-catenin, which activates the transcription of target genes in association with T-cell-specific factors (Tcfs). Although LIF/STAT3 signaling is crucial for mouse ES maintenance, it does not appear to have a role in hES propagation. Sato and collegues were able to demonstrate that activation of the Wnt pathway is sufficient to maintain self-renewal of both mouse and human ES cells. They used 6-bromoindirubin-3′-oxime (BIO), a pharmacological intracellular inhibitor of GSK3, to activate the Wnt pathway in both mouse and human ES cells. BIO-treated ES cells display nuclear accumulation of β-catenin, which demonstrates that ES cells are capable of transducing Wnt signaling. Additionally, BIO-activated Wnt signaling results in sustained expression of the pluripotent markers Oct-4, *Nanog*, and Zfp-42 *Rex1*. Notably, in short-term cultures, BIO can substitute for LIF and MEF-conditioned medium in the propagation of pluripotent mouse and human ES cells respectively.

LIF and Wnt Signaling Converge on a Common Target

Stat3 is required for the gp-130 dependent activation of the *c-Myc* gene in pro-B cell lines. Catwright and collegues showed that *c-Myc* is a STAT3 target gene in ES cells as well. STAT3 binding to *c-Myc* promoter depends on LIF. However, MYC protein is degraded upon GSK3-dependent phosphorylation of Threonin 58 (T58). ES cells that conditionally express a T58A mutant protein can be propagated in the absence of LIF, while cells expressing a dominant negative MYC protein with decreased activity are prone to differentiation. These results indicate a requirement for a stable form of MYC, and consequently for inhibition of T58 phophorylation during ES self-renewal, which is consistent with the observation that differentiating ES cells display an increase in GSK3 activity as early as one day after LIF removal. LIF/STAT3 and Wnt signaling converge thus on *c-Myc*, by ensuring respectively gene transcriptional activation and stabilization protein.

THE TGFβ SIGNALING PATHWAY

The TGFβ superfamily of ligands includes nearly 30 proteins in mammals, e.g., TGFβ, Activins, Nodal, GDF, and BMPs. Smad1/5/8 transduce signals from Bmps and GDFs ligands, whereas Smad2/3 transduce signals from TGFβ,

Activins, and Nodal. Upon activation by phosphorylation and association with a common Smad4, the receptor-activated Smads translocate to the nucleus to regulate gene expression in concert with other transcription factors. Smad6 and Smad7 are inhibitory SMADs.

BMP4

As described before, LIF is sufficient to guarantee mouse ES self-renewal in a serum-enriched growth environment. However, when ES cells are cultivated in serum-free medium containing LIF, they tend to spontaneously undergo neural differentiation. This indicates the existence of one or multiple growth factors contained in fetal calf serum (FCS), which cooperate with LIF in the maintenance of ES cell pluripotency. Ying and collegues identified BMP4 as the growth factor that acts in combination with LIF to sustain self-renewal, and to prevent/block neural differentiation of ES cells in serum-free cultures. This was an unexpected result, as Bmp4 alone promotes differentiation into non-neural-fates. Bmp4 induces phosphorylation of Smad1/5/8 which, in association with Smad4, translocates into the nucleus to activate expression of the Inhibitor of differentiation (Id) protein. This in turn blocks neural differentiation by functionally antagonizing neurogenic bHLH factors (Figure 6-3). Forced expression of Id eliminates BMP or serum dependence and allows self-renewal in the presence of LIF alone. However, Id-expressing ES cells cannot differentiate into the neural lineages, further confirming the Bmp-4 induced lineage-specific block. When added to hES, BMP4 triggers differentiation into primitive endoderm and trophoblast lineages.

Activin/Nodal

In an alternative study, Ogawa *et al*. showed that in serum-free culture exogenous activin or Nodal, but not Bmp or Tgfβ, significantly enhance ES propagation without affecting pluripotency. Over-expression of Smad7, a specific inhibitor of both activin-Nodal and Bmp signaling, decreases ES proliferation, whereas Smad6 overexpression, which specifically inhibits Bmp signaling, does not elicit such an effect. Although the function of Smad7 in mouse embryogenesis is still unknown, these data are supported by the analysis of Nodal function *in vivo*. Nodal ablation leads, in fact, to a substantial reduction of the embryo epiblast size and to low *Oct4* expression at 6.5 dpc. The results presented by Ogawa *et al*. are in contradiction with those reported by Ying *et al*. This inconsistency is probably due to the fact that different serum-free culture conditions were used in the two studies.

Activation of TGFβ/activin/nodal signaling via SMAD2/3 was also shown to be required for maintenance of the undifferentiated state of human ES cells. Additionally, hES differentiation is accompanied by a decrease in SMAD2/3 signaling and by activation of SMAD1/5 signaling, which is instead inhibited in undifferentiated cells. Activin, MEF conditioned medium and BIO all sustain high levels of Smad2/3 phosphorylation and *Oct4* expression in hES cells, indicating that Smad2/3 activation is the common denominator to the three signaling pathways in ensuring hES cell identity.

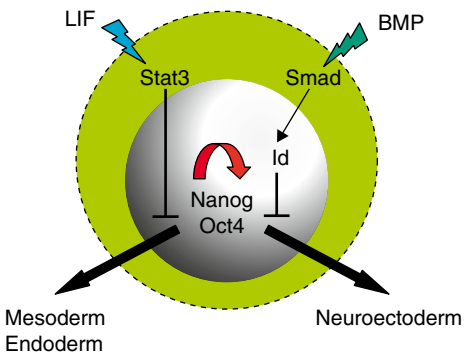


FIGURE 6-3 In serum-free conditions BMP and LIF collaborate in maintaining ES cell self-renewal by suppressing multi-lineage differentiation. Bmp accomplish this role by inducing Id gene expression via the Smad pathway. Adapted from Ying *et al.*, 2003.

bFGF and TGFβ Signaling in hES Cells

Human ES cells can be maintained in an undifferentiated status in the presence of basic fibroblast growth factor (bFGF) when grown on fibroblast feeder layers or in fibroblast-conditioned medium. They can be cultured in feeder-free conditions as well, but only in the presence of a combination of bFGF and Noggin, an inhibitor of Bmp4. Strikingly, in order to condition medium for hES growth/self-renewal, MEF must also be cultured with bFGF. Genome-wide expression profiling of supportive (grown with bFGF) and non-supportive MEF cells (grown without bFGF) revealed that bFGF stimulation regulates the expression of key members of the TGF-β pathway, including Inhba, Tgfb1, Grem1 (a Bmp4 inhibitor), and Bmp4. In particular, Tgfb1, Inhba, and Grem1 are up-regulated, whereas Bmp4 is down-regulated in bFGF-treated MEF. As these same genes are also targets of bFGF regulation in hES cells, these results suggest that FGF and TGF-β signaling operate in a concerted manner to achieve hES cell self-renewal.

THE PI3K/AKT SIGNALING PATHWAY PARTICIPATES IN ES CELL PROLIFERATION, SURVIVAL, AND PLURIPOTENCY

PI3K phosphorylate phosphatidylinositol-3,4-diphosphate (PIP2) into the second messanger PIP3, which signals to downstream effectors including AKT/PKB, a serine/threonine kinase implicated in the regulation of cell cycle

progression, cell death, and tumorigenesis. The lipid phosphatase and tumor suppressor PTEN antagonizes AKT activation by dephosphorylating PIP3 into PIP2. Involvement of the PI3K signaling pathway in ES cell propagation was first proposed through the analysis of ES cell behavior after PTEN deletion. PTEN-null ES cells display increased proliferation rate/cell survival and tumorigenicity. They also have enhanced AKT/PKB activity, and consequently elevated amounts of PIP3. In contrast, double null pten/AKT ES cells revert to the proliferation rates of wild type ES cells, indicating that *Akt* up-regulation is the main cause of the tumorigenic/proliferation phenotype observed in PTEN-null ES cells. Treatment of ES cells with the PI3K inhibitor LY294002 inhibits proliferation and induces cell cycle arrest at G0/G1. Additionally, genetic deletion of the small GTPase Eras, which is an activator of PI3K/AKT signaling, reduces the endogenous proliferative and tumorigenic potential of ES cells.

The PI3K signaling pathway is activated and/or induced by LIF treatment, as assessed by measurements of PI3K-dependent modifications, e.g., phosphorylation of serine 473 on PKB and of serine 21/9 on GSK3β. Chemical inhibition of PI3K and AKT induces differentiation of, respectively, mouse and human ES cells by enhancing activation of the MAP kinases ERK1/2. This result suggests that, besides having an impact on ES proliferation, the PI3K/AKT signaling cascade may have a potential role in the maintenance of ES cell pluripotency. Watanabe and collegues showed that a myristoylated active form of AKT is capable of maintaining mouse and primate ES cells undifferentiated in the absence of LIF and MEF cells, respectively. AKT/PKB inhibits GSK3 through the phosphorylation of Ser-9. However, AKT signaling maintains cells in the undifferentiated state independently of Wnt/β catenin and STAT3/ ERK signaling, as myr-AKT expressing ES cells show neither nuclear β-catenin accumulation nor increased Stat3 phophorylation. AKT promotes a G1 to S phase transition by regulating cyclin D expression, and by facilitating formation of cyclin/CDK complexes. ES cells are unique for going through a very short G1 phase, owing to absence of the cell cycle inhibition mediated by the CDK inhibitor Ink4 and tumor suppressor RB. Since cell cycle inhibitory mechanisms are acquired during differentiation, then AKT may exert its role on pluripotency by blocking the ability to gain those inhibitory mechanisms. However, cyclin D overexpression does not maintain ES cells in an undifferentiated state, indicating that regulation of cell cycle alone cannot explain the effect of AKT on ES pluripotency.

THE NEW PARADIGM OF EMBRYONIC STEM CELL RENEWAL

Unlimited proliferation of mouse ES cells *in vitro* is guaranteed by the presence of LIF and BMP or serum. Interestingly, ICM-derived cells remain pluripotent in the embryo *in vivo*, even when the LIF/Stat3 signaling is genetically inactivated. The explanation of this apparent paradox was finally given by the investigations conducted in Smith's laboratory. According to the model proposed in the study published in May 2008, LIF and serum or BMP do not have an instructive role in stem cell renewal, but rather they function by imposing a block on differentiation-inducing signals.

Since differentiation is characterized by auto-inductive stimulation of the MAPK/ERK pathway by FGF4, SU5402 and PD184352 were used to inhibit FGF receptor tyrosine kinases and the ERK cascade, respectively. Undifferentiated ES cells can be passaged in the presence of both inhibitors, although occasional neuronal differentiation is observed. Low levels of phospho-ERK are detected when cells are grown with SU5402 and PD184352. However, the two inhibitors compromise the viability and growth rate of ES cells, as indicated by poor survival at clonal density and after passage. Alone, CHIR99021, a selective inhibitor of GSK3, enhances survival at low density, but also induces non-neural differentiation. Crucially, the combination of the three inhibitors supports *de novo* ES derivation and efficient propagation of undifferentiated ES cells even at low cell density. Additional validation to the role that FGF signaling has on stem cell renewal is provided by fgf4-null ES cell propagation in CHIR99021 alone. Thus, at the ground state, ES cells are doomed to spontaneous differentiation by auto-inductive stimulation of the ERK pathway. Self-renewal is ensured by elimination of ERK signaling. SU5402 and PD184352 enable self-renewal by inhibiting ERK activation; LIF and BMP counteract signaling emanating from activated ERK1/2 (see Figure 6-4).

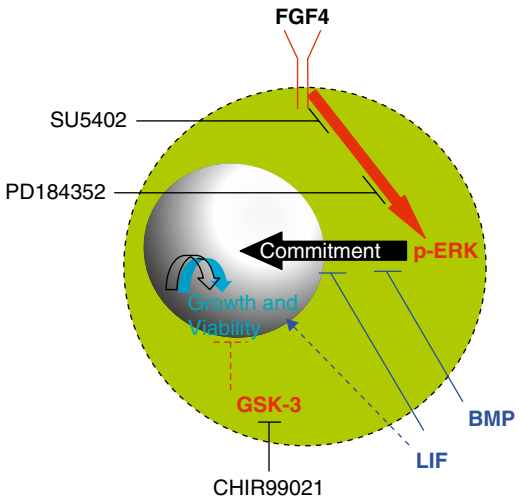


FIGURE 6-4 Schematic representation of the replicative pluripotent state in different culture conditions. Phospho-ERKs are the source of differentiation-inducing signals. Self-renewal results from either the upstream chemical inhibition of ERK activation by SU5402 and PD184352 or from LIF/Bmp-mediated inhibition of signaling emanating from phospho-ERKs. In the first scenario inhibition of GSK3 is necessary in order to augment ES cell growth and viability. Adapted from Ying *et al.*, 2008.

INTRINSIC DETERMINANTS OF PLURIPOTENCY

The phenotype of a particular cell type is the culmination of a well-defined and unique gene expression pattern, characterized by the activation of some genes and repression of others. Therefore, elucidation of the molecular basis of pluripotency is based primarily on the identification of the key transcription factors involved in regulating gene expression at the pluripotent "state." In a very simplistic model, genes that promote and maintain an undifferentiated cellular state would be expressed in pluripotent cells, while those that are activated during stem cell differentiation would be repressed. Conversely, in terminally differentiated cells, pluripotency-related genes would be silenced, whereas those required for the differentiated cell state would be expressed. Elements that influence the transcriptional regulation of gene expression include epigenetic modifications of the genome, such as methylation and histone deacetylation, which determine what types of interactions are allowed between DNA and transcription factors and modulating cofactors. A combination of cell culture and gene ablation studies in mouse embryos has led to the identification of the intracellular determinants of ICM and ES pluripotency.

OCT4 IS A KEY TRANSCRIPTION FACTOR FOR PLURIPOTENCY

Oct4 is a transcription factor belonging to the class V of POU factors. The POU family of transcription factors binds to the octamer motif ATGCA/TAAT found in the regulatory domains of cell type-specific as well as ubiquitous genes (ATGCAAAT). POU factors have a common conserved DNA binding domain, called the POU domain, which was originally identified in the transcription factors *Pit1, Oct1, Oct2*, and Unc86. The POU domain is comprised of two structurally independent subdomains—the POU specific domain (POU_S) and the homeodomain (POU_H)—connected by a flexible linker of variable length.

The POU domain of *Oct4* is characterized by several properties that confer the OCT4 protein an impressive versatility in the operational mode of transcriptional regulation:

- Flexible amino acid-base interactions allowing recognition of moderately variable cognate DNA elements;
- Variable orientation, spacing and positioning of DNA-tethered POU subdomains relative to each other on the DNA, as evidenced by the different arrangement on the Palindromic Oct factor Recognition Element (PORE);
- Cooperative binding of the two subdomains to the DNA;
- Interaction with other transcription factors and regulatory modulators;
- Post-translational modifications modulating OCT4 transactivation in various cell types.

The POU domain binds to DNA via interaction of the third "recognition" helix of the POU_H with bases in the DNA major groove at the 3′A/TTTA portion of the octamer site. The POU_H domain is structurally similar to other homeodomains. The POU_S domain exhibits site-specific, high-affinity DNA binding and bending capability. Both the POU_S and POU_H subdomains function as structurally independent units with cooperative high-affinity DNA-binding specificity. Functional cooperation between the two subdomains may occur indirectly via the DNA by overlapping base contacts from the two subdomains.

In addition to the DNA binding function, both the POU_H and POU_S subdomains can participate in protein–protein interactions. In ES cells, OCT4 activates gene transcription irrespective of the octamer motif distance from the transcriptional initiation site. However, OCT4 can transactivate only from a proximal location in differentiated cells. In this scenario, interaction between the adenovirus E1A or human papillomavirus E7 protein and OCT4 POU domain is sufficient for OCT4 to elicit transcriptional activation from remote binding sites. E1A and E7 proteins would therefore mimic unidentified embryonic stem cell specific coactivators that serve a similar function in pluripotent cells.

Two domains spanning the N- and C-terminal portion of Oct4 protein define the transactivation capacity of the POU transcription factor. The N-terminal region (N domain) is a proline- and acid residue-rich region, whereas the C-terminal domain (C domain) is a region rich in proline, serine, and threonine residues. The N-terminal domain can function as an activation domain in heterologous cell systems. However, the C-terminal domain of OCT4 exhibits a POU domain mediated cell type-specific function. Intramolecular interactions between the POU domain and C-terminal domain may lead to cell type-specific interactions with different cofactors or kinases. An interesting regulation model of the C-domain predicts that association between the OCT4 POU domain and other factors can alter the phosphorylation status of the protein and ultimately modulate the activity of the C-domain.

OCT4 EXPRESSION

In the Mouse

Oct4, otherwise designated *Oct3* or *POU5F1*, is a maternally-inherited transcript that is developmentally regulated in mice. It is expressed at low levels in all blastomeres until the four-cell stage, at which time the gene undergoes zygotic activation resulting in high OCT4 protein levels in the nuclei of all blastomeres until compaction. After cavitation, *Oct4* is maintained only in the inner cells (ICM) of the blastocyst and is down-regulated in the differentiated trophectoderm (TE). Following implantation, *Oct4* expression is restricted to the primitive ectoderm (epiblast), although it is transiently expressed at high levels in cells of the forming hypoblast (primitive endoderm). During gastrulation, *Oct4* expression

becomes down-regulated in the epiblast in an anterior to posterior manner. From 8.5 dpc on, *Oct4* becomes restricted to precursors of the gametes or PGCs. *Oct4* is also expressed in undifferentiated mouse ES, EG, and EC cell lines. ES and EC cell treatment with the differentiation-inducing agent retinoic acid (RA) induce rapid *Oct4* down-regulation in both cell types. *Oct4* is not expressed in differentiated tissues. Recently, a very low amount of *Oct4* messenger was detected in multipotent adult precursor cells (MAPCs) and human breast cancer cells.

In Humans

Oct4 is highly expressed in ICM cells relative to TE cells in discarded human embryos. It is also expressed in pluripotent human EC and ES/EG lines.

OCT4 ACTIVITY IN ASSAYS

Gene inactivation experiments clearly indicate that *Oct4* plays a determinant role in the specification of mouse pluripotent cells. However, *Oct4* function is not confined to pluripotent cells. Extrapolation of the data obtained by manipulating *Pou5f1* expression in ES cells indicates that *Oct4* is a regulator of cell fate in all three tissues of a pre-implantation embryo.

Embryos With No Oct4

Oct4-deficient embryos die at the peri-implantation stage and form implantation sites that contain trophoblastic cells but are devoid of yolk sac or embryonic structures. The embryos develop up to the 3.5 dpc blastocyst stage, with an unaltered number of cells in the ICM or trophoectoderm as compared to wild type embryos, suggesting that in the absence of OCT4 protein, cell proliferation is not affected during early stages of development. *In vitro* cultures of ICM cells isolated from the *Oct4* null blastocysts are devoid of pluripotent and extra-embryonic endoderm cells, but do contain trophoblastic giant cells.

These results indicate that the ICM of *Oct4*-deficient blastocyst is defined only by the stereo arrangement of non-pluripotent cells. These findings lead to two main conclusions:

1. *Oct4* is essential in the establishment of the ICM pluripotency;
2. In the absence of *Oct4*, interior cells of the pre-implantation embryo are diverted to a trophoectoderm fate.

ES Cells With Low and High Levels of Oct4

Based on *Oct4* expression levels during the first two events of lineage commitment in the early embryo, it is not entirely surprising that a critical amount of *Oct4* has recently been reported to be crucial for the maintenance of ES cell self-renewal.

ES cells containing a bacterial genetic system for the conditional expression and repression of Oct4 transgene allowed researchers to determine the cellular phenotype linked to variable levels of OCT4 protein. Briefly, ES cells in which one or both endogenous *Oct4* alleles had been inactivated were transfected with constructs for a tetracycline-regulated transactivator and a responsive *Oct4* transgene, whose precise level of expression was modulated by variable amounts of the antibiotic.

A 50% decrease in the endogenous *Oct4* levels relative to that of undifferentiated ES cells resulted in the commitment of ES cells to trophoectoderm lineages, containing both proliferating and endo-duplicating giant cells based on the culture conditions. This result is consistent with the phenotype of *Oct4* deficient embryos and the observation that trophoectoderm differentiation of outer cells of the morula is accompanied by *Oct4* down-regulation (Okamoto, 1990; Rosner, 1990; Schöler, 1990a).

However, a 50% increase beyond the threshold level of *Oct4* leads to the concomitant differentiation of ES cells into extra-embryonic endoderm and mesoderm. Interestingly, LIF withdrawal leads to the specification of the same lineages. Smaller changes in *Oct4* level (whether increases or decreases) do not affect ES cell self-renewal. In conclusion, the precise level of OCT4 protein governs commitment of embryonic cells along three distinct cell fates (self-renewal, trophectoderm, or extra-embryonic endoderm and mesoderm) (see Figure 6-5).

The conditional *Oct4*-null ES line proved to be a useful tool to establish which domains of the OCT4 protein are sufficient to maintain ES cells in complementation assays. The complementation assay was based on the ability of a proper *Oct4* molecule to rescue the self-renewal capability of cells that would otherwise differentiate due to Oct4 down-regulation. OCT4 is the only POU protein that has the ability to rescue the self-renewing phenotype, as OCT2 or OCT6

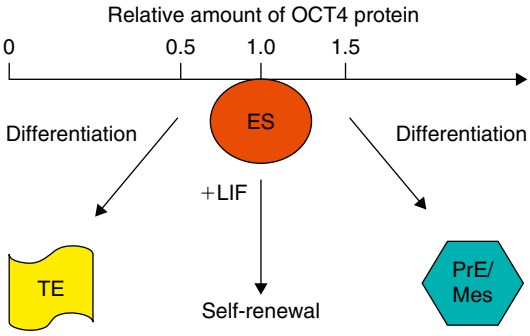
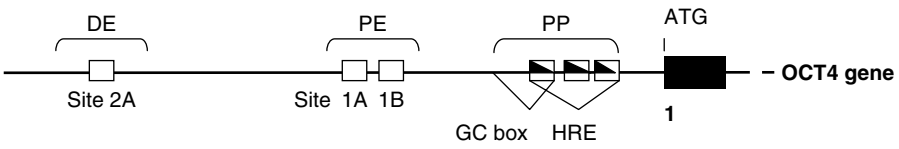


FIGURE 6-5 Model depicting the consequences of OCT4 variable protein concentration on ES cell fate. TE stands for trophoectoderm; PrE/Mes stands for primitive endoderm/mesoderm.



Distal enhancer	Proximal enhancer
Pre-implantation embryo Oocyte through morula stage Post-implantation embryo: Primordial germ cells	Post-implantation embryo: Embryonic ectoderm
Embryonic stem cells Embryonic germ cells F9 embryonal carcinoma cells	P19 embryonal carcinoma cells

FIGURE 6-6 Representation of the OCT4 gene. Open and partially filled boxes indicate conserved regions included within the proximal/distal enhancer and the proximal promoter. The black box indicates the first exon of the gene. Cell and tissue specificity conferred by the two enhancers is also described.

has no effect on cell fate in this system. A truncated OCT4 protein containing the OCT4 POU domain and either the N- or C-terminal domain can support ES cell self-renewal as the wild-type counterpart. Furthermore, gene expression analysis revealed that OCT4 transactivation domains, although equivalent in sustaining the undifferentiated stem cell phenotype, elicit activation of different target genes. It would be interesting to determine the consequences of either the N- or C-domain deletion on embryo development *in vivo*.

REGULATION OF OCT4 EXPRESSION

Use of *Oct-4/LacZ* transgenes has allowed the identification of two distinct enhancer elements, which are driving the cell type-specific expression of *Oct4* in a reciprocal fashion. The distal enhancer or DE, located approximately 5 Kb upstream of the promoter, regulates *Oct4* expression in pre-implantation embryos (morula and ICM), PGCs, and in ES, F9 EC, and EG cells. However, the DE is inactive in cells of the epiblast. The proximal enhancer or PE, located approximately 1.2 Kb upstream, directs *Oct4* expression in the epiblast, including *Oct4* down-regulation in the anterior to posterior direction observed during gastrulation, and in P19 EC cells (see Figure 6-6). Two similar inverted elements, the 2A site within the DE and the 1A site within the PE, are bound by transcription factors in undifferentiated ES and EC cells *in vivo*. Upon RA-induced differentiation, protection of these two areas is lost. These data suggest that these elements are involved in regulating *Oct4* transcription probably through local signals and/or cell type-specific factors.

The *Oct4* gene comprises a TATA-less promoter, called PP, containing a GC-rich Sp1-like sequence and three hormone response element half sites—(HRE) R1, R2 and R3. Several nuclear receptors, retinoid acid receptors and the orphan receptors COUP-TFI/II, SF1, LRH1 and GCNF, have been shown to be involved in the regulation of Oct4 expression *in vitro*. However, knockout mouse models have substantiated such a role only for *LRH1 and gcnf*.

ChIP analysis was employed to demonstrate that binding of LRH1 to the PE and PP regions of the Oct4 gene occurs in undifferentiated ES/P19 cells and is lost upon RA induced differentiation. LRH1 binding sites drive the expression of *Oct4*-PE-PP-luciferase constructs in undifferentiated P19 cells. Additionally, *lrh1*-null ES cells, display a much quicker loss of *Oct4* expression upon LIF removal than is observed for wt ES cells. *In vivo*, *LRH1* is expressed in the ICM and visceral endoderm of the blastocyst, and at the egg cylinder stage throughout the whole embryo. *LRH1* deficient embryos die at 6.5 dpc and display no detectable Oct4 mRNA, which indicates that *LRH1* is specifically required to maintain *Oct4* in the embryo epiblast.

During RA-induced differentiation of P19 cells, Oct4 down-regulation is temporally followed by *gcnf* up-regulation. GCNF binding to the promoter R2 repeat represses transcription driven by the proximal enhancer–promoter (PEP) in P19 cells. Analysis of *gcnf*-deficient embryos has emphasized the impact of *gcnf*-induced repression of the *Oct4* gene. Low *gcnf* expression is detected in the whole mouse embryo at 6.5 dpc. At 7.5 dpc, increasing *gcnf* and decreasing *Oct4* mRNA levels are observed in neural folds and at the posterior of the embryo. As mentioned above, at the end of gastrulation *Oct4* is maintained only in PGCs at the base of the allantois. In 8.0–8.5 dpc *gcnf*-deficient embryos, Oct4 mRNA is detected in the putative hindbrain region and posterior of the embryo, which indicates that *gcnf* ablation leads to loss of *Oct4* repression in somatic cells or loss of GCNF-induced *Oct4* restriction in the germline.

The same phenotype is observed in *gcnf*-deficient mice containing a targeted deletion of the DNA binding domain of GCNF. *gcnf*-null ES cells maintain expression of *Oct4* (and pluripotent genes) during a six-day time course of RA treatment. Taken together, these findings strongly point towards GCNF as an essential regulator of *Oct4* repression both *in vivo* and *in vitro*.

DNA methylation constitutes an important mechanism of gene expression regulation during embryogenesis. A global loss of DNA methylation occurs during cleavage prior to the 16-cell stage. Following implantation, a wave of *de novo* DNA methylation occurs in all genes, except those containing CpG islands. *In vivo* analysis of the PE-P region of the *Oct4* gene reveals that *Oct4* is not methylated from the blastocyst stage up to 6.25 dpc, a time during which other genes undergo *de novo* methylation. Interestingly, a cis-specific demethylation element exists within the *Oct4* PE. Mutational analysis shows that site 1A within PE is involved in preventing methylation. The presence of this cis-specific demodification element causes demethylation of sequences that had been methylated prior to their introduction into EC cells, and protects from *de novo* methylation *in vivo*. Interestingly, methylated PE sequences induce a decrease in transcriptional activation both of a reporter gene and the endogenous *Oct4* in P19 cells. According to the model proposed by Gidekel and Bergman, binding of trans-acting factors to the PE element offers protection against *de novo* methylation, which can begin soon after gastrulation, when these factors are down-regulated. At the end of gastrulation, these factors would be present only in the germ cell lineage, the sole lineage to express *Oct4* by 8.5 dpc.

As for the PE, the *Oct4* promoter region spanning between −562 and the putative ATG was shown to contribute to the epigenetic regulation of *Oct4* transcription. The region contains 16 CpG sites, which are unmethylated in undifferentiated ES cells and display increased methylation status upon RA treatment. In the absence of GCNF, *Oct4* promoter is hypomethylated both in differentiating ES cells and in 8.5 dpc embryos. Using a two-hybrid assay, Gu *et al.* found the methyl DNA binding domain proteins MBD3 and MBD2 as GCNF-interacting factors whose GCNF-dependent recruitment to *Oct4* promoter is necessary for Oct4 repression and methylation. *Mbd3* and *Mbd2* belong to the MeCP1 and NurD (Nucleosome remodeling and histone deacetylation) transcriptional repression complexes. In agreements with these results, *Mbd3*-deficient ES cells display LIF-independent self-renewal, and a compromised *in vivo* differentiation potential. *In vitro* they can only differentiate upon RA treatment.

OCT4 TARGET GENES

Human chorionic gonadropin (hCG) is required for implantation and maintenance of pregnancy. It is secreted by the trophectoderm of peri-implantation blastocyst. *Oct4* has been shown to silence the expression of α and β subunit genes of hCG in human choriocarcinoma cells. *Oct4* binding to a unique octamer motif (ACAATAATCA) in the hCGβ −305/−249 promoter region considerably reduces both hCGβ mRNA and protein levels in JAr cells. Although Oct4 is a potent inhibitor of hCGα expression, no octamer binding site has been identified in the promoter region of hCGα.

Like CG in humans, tau interferon (IFN-τ) is needed to prevent regression of the maternal *corpus luteum* during the early stages of pregnancy in ruminant species. In cattle, members of the multigene IFN-τ family are expressed in the TE from the blastocyst stage up to the beginning of the placentation process. IFN-τ expression is promoted by an Ets-2 enhancer, which is located in the promoter region of the gene.

Unlike *Oct1* or *Oct2*, *Oct4* specifically interferes with Ets-2-induced activation of *IFN-τ* promoter in JAr cells. These data, taken together with the observation that reduced Oct4 protein level triggers differentiation of murine ES cells into trophoectoderm, suggest that silencing of *Oct4* in the TE is a prerequisite for up-regulation of *hCGs* and *IFN- τ*.

The *Rex1* gene, which encodes a zinc finger protein, is expressed at high levels in ES and EC cells, and is down-regulated upon RA treatment. Both processes are mediated by the octamer motif located in the promoter region of *Rex1*. Low levels of OCT4 protein are sufficient to activate *Rex1* promoter in P19 RA-differentiated cells, whereas high OCT4 or OCT6 level inhibit transcription in F9 cells. Distinct Oct4 protein domains elicit the observed effects, suggesting different molecular mechanisms of *Oct4* mediated transcriptional activation and repression.

Expression of the platelet-derived growth factor α receptor (PDGFαR) in undifferentiated human EC Tera2 cells depends on a canonical octamer motif within the gene promoter. Mutation of the octamer site has been shown to decrease the promoter activity and *PDGFαR* expression.

OCT4 DOES NOT PLAY *SOLO:* SOX2

Sox2 belongs to the Sox (*S*ry-related HMG b*ox*-containing) family of proteins that bind to DNA through the 79-amino acid HMG (high mobility group) domain. In contrast to most DNA-binding proteins, which access to DNA through the major groove, the HMG box interacts with the minor groove of the DNA helix and, as a consequence, induces a dramatic bend in the DNA molecule. As a result of this energetically high cost interaction, Sox proteins bind to DNA with a high dissociation constant.

Sox2 Expression

Sox2 is co-expressed with *Oct4* in the ICM of preimplantation embryos, ES cells, EC cells, and germ cells. At the

egg cylinder stage, *Sox2* is expressed both in the epiblast and in the adjacent extra-embryonic ectoderm (ExE). In 7.5 dpc embryos *Sox2* mRNA marks the chorion and the presumptive neuroectoderm.

Sox2 Function *In Vivo*

Analysis of *Sox2*-deficient embryos supported the hypothesis that functional association between OCT4 and SOX2, previously observed in cultured cells, constitutes a new paradigm of gene activation in the early embryo as well (see following paragraph). *Sox2*-deficient embryos die shortly after implantation at around 6.0 dpc, with implants lacking *Oct4*-positive epiblast cells and having disorganized extra-embryonic tissues. Post-implantation lethality cannot be attributed to lack of *Sox2* expression in ExE, as wild-type ES cell can rescue development of *Sox2*-deficient embryos up to E7.5–7.75.

Immunosurgically isolated *Sox2*-null ICMs are diverted to a trophoectodermal and endodermal fate. In *Sox2*-deficient blastocysts, very low *Oct4* expression can be detected by RT-PCR, indicating that a pluripotent ICM can be specified but not maintained (see Figure 6-7). However, complete knockout of the *Sox2* gene is not practicable during early development, as maternal SOX2 protein persists throughout pre-implantation development. Failures in establishing *Sox2*-null ES cell lines confirm *Sox2* role in the maintenance of pluripotent stem cells.

Sox2 and OCT4 Partnership

Regulation of the *fgf*4 and *utf1* genes provided insight into an OCT4-induced gene activation model based on parternship with the transcription factor *Sox2*.

Distinct regulatory elements govern *fgf4* gene expression in the mouse ICM, myotomes and developing limb.

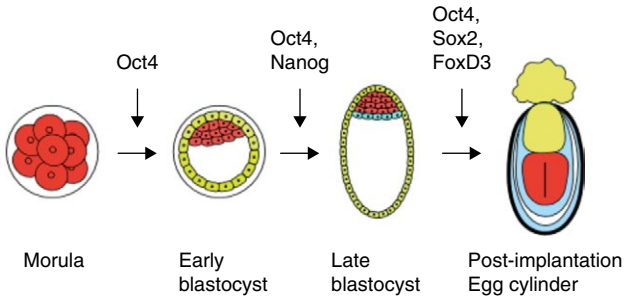


FIGURE 6-7 Model relative to the roles played by Oct4, Nanog, Sox2, and FoxD3 during early mouse development. Oct4 is essential in the first embryonic lineage specification, whereas Nanog function is to prevent endoderm differentiation of the ICM cells. Sox2 and FoxD3 are essential in the maintenance of a pluripotent epiblast. Depicted in red are the pluripotent compartments of the embryo, e.g., ICM and epiblast. Blastocyst trophoectoderm and trophoectoderm-derivatives of the post-implantation embryo are depicted in yellow. Blastocyst primitive endoderm and visceral/parietal endoderm of the post-implantation embryo are depicted in light blue.

fgf4 expression in ICM and in ES/EC cells is conferred by a distal enhancer localized in the 3′ UTR of the gene. *Oct4* (or *Oct1*) and SOX2 bind to the respective cognate sites in the "embryonic" enhancer and form a unique ternary complex that elicits strong synergistic transcriptional activation. Formation of an active ternary complex is highly dependent on the spatial arrangement of the adjacent Sox and Oct binding sites on the DNA, since insertion of three base pairs between the Sox and Oct recognition sequences of the *fgf4* gene severely impairs the enhancer function. The C-terminal domains of both SOX2 and OCT4 contribute to the functional activity of Sox2/OCT4 complex. The synergistic action of SOX2 and OCT4 results from two distinct yet concerted events. The first event involves cooperative binding of SOX2 and OCT4 to the DNA via their respective DNA binding domains. The tethering of each factor to the enhancer region on fgf4 ameliorates the intrinsic activity of the activation domains of each protein. Upon formation of the ternary SOX2/OCT4 complex, novel DNA–protein and protein–protein interactions induce conformational changes that may lead to activation of latent domains and constitute a new, distinct platform for the recruitment of other co-activators. In line with this model, synergistic activation of *fgf*4 transcription by SOX2 and OCT4 in HeLa cells is mediated by p300, a potential bridging factor, which should promote enhancer–promoter interactions. Notably, the embryonic fgf4 enhancer sequences are conserved in the mouse and human genes.

Embryonic stem cell-specific expression of *utf1* is also regulated by the synergistic action of SOX2 and OCT4. The binding sites for these two factors are arranged with no intervening spacing in the second intron of the *utf1* gene. Interestingly, one base difference in the canonical octamer binding sequence enables the recruitment of an active OCT4/SOX2 complex and prevents binding of a transcriptionally inactive complex containing OCT1 or OCT6. The sequence ACTAGCAT (canonical sequence: ATTA/TGCAT) is recognized specifically by OCT4, while OCT1 and OCT6 can bind to it only by exploiting half of the 3′ adjacent Sox site (AACAATG). The OCT4 POU_H domain is essential in ensuring unique DNA binding ability on the *utf1* octamer consensus sequence.

The F-box containing protein 15(*Fbx*15) is expressed predominantly in ES cells. Embryonic expression of *Fbx15* results from the cooperative binding of OCT4 and SOX2 to cognate sites juxtaposed in the *Fbx15* ES specific enhancer.

Zfp206, a transcription factor that is highly expressed in mouse and human ES and down-regulated during ES differentiation, is another example of OCT4/SOX2 target genes.

OCT4–OCT4 Dimers and Sox2

The structural flexibility of the linker sequence between the two independent POU subdomains endows OCT4 with the

ability to form homodimers on specific DNA sequences. A novel PORE, composed of an inverted pair of homeodomain-binding sites and separated by five base pairs (ATTTGaaatgCAAAT), has been identified in first intron of the EC/ES cell-specific *opn* gene enhancer.

OPN is an extra-cellular phosphoprotein, which modulates cell migration and adhesion of the primitive endoderm cells by binding to specific integrins. In the mouse embryo, *opn* is co-expressed with *Oct4* in ICM, forms primitive endoderm at 4.0 dpc, and is down-regulated in the epithelial hypoblast of E 4.5 embryos. In EC and ES cells, *opn* expression is inversely correlated with SOX2 protein levels.

The POU transcription factors OCT1, OCT6, and OCT4 bind to the DNA PORE element as monomers, homo-, and heterodimers. Oct4 monomer does not elicit transcriptional activation via the PORE in EC cells. Rather, activation of gene transcription is highly dependent on OCT4 dimerization on the PORE. In fact, mutation in the palindromic element of either home domain-binding sites drastically impairs dimerization and transcriptional activation. Interestingly, SOX2 represses OCT4 mediated *opn* gene transactivation by binding to the cognate site adjacent to the PORE in the *opn* intron. SOX2 may interfere with OCT4 dimer formation.

Dimerization on the PORE creates a specific conformational surface that may be suitable for interaction with unidentified co-activators. In fact, OCT1/ or OCT2/PORE dimers can interact and synergize in transcriptional activation with the lymphoid specific co-activator OBF-1. According to a model of the PORE OCT4 dimer structure, Isoleucine 21 (I21) of the POU_S and Serine 107 (S107) of the POU_H of two distinct OCT4 molecules make specific contact in the PORE dimer interface. Mutation of either residue impairs dimerization on the PORE element. S107 corresponds to a conserved phosphorylation site identified in OCT1 and PIT1. Phosphorylation of this serine has been shown to influence OCT1 binding to DNA and may provide an additional level of regulation of OCT4 dimer activity. Mutation of S107 into glutamate might structurally mimic a phosphorylated serine. To date, *opn* is the only known *Oct4* target gene to be transcriptionally regulated by OCT4 dimers. Mutant mice with impaired dimerization of the OCT4 protein might be valuable in identifying new Oct4 dimer target genes and their function during early development.

FOXD3

FoxD3, a transcription factor belonging to the forkhead family, is expressed in mouse and human ES cells, and during mouse embryogenesis in the epiblast and neural crest cells. FoxD3-deficient blastocysts present a regular pattern of *Oct4*, *Sox2*, and *fgf4* expression before implantation. Nevertheless, mutant mouse embryos die at 6.5 dpc and display a reduction of the epiblast, extension of the proximal extra-embryonic endoderm and ectoderm tissues, and lack of primitive streak. In addition, FoxD3-deficient ICMs fail to expand after prolonged culture *in vitro*. These results suggest that FoxD3 is essential for epiblast maintenance, but is not required for differentiation of extra-embryonic tissues (Figure 6-7).

NANOG

In 2003 Chambers and Matsui reported on the identification of *Nanog* as a new member of the embryonic stem cell stage. Like OCT4, NANOG protein contains a homeodomain, which shares a maximum of only 50% amino acid identity with members of the NK2 family of homeoproteins. This makes NANOG a unique divergent homeodomain protein.

Nanog Expression

Nanog is found to be expressed in a quite restricted pattern. *In vivo*, *Nanog* expression is first detected in the interior cells of compacted morulae and then in the ICM and the epiblast of a pre-implantation blastocyst, but it declines prior to implantation. Following implantation, *Nanog* transcripts can be found in a subset of epiblast cells and at E11.5 in post-migratory germ cells (PGCs). *In vitro*, *Nanog* marks all pluripotent cell lines, from ES (both murine and human) to EG and EC. Hence the name Nanog, after the Celtic land of the ever-young Tir nan Og.

Nanog Function *In Vivo* and *In Vitro*

Nanog-deficient embryos die soon after implantation due to a failure in the specification of the pluripotent epiblast, which is diverted to an endodermal fate. Similarly, *Nanog* ablation in ES cells causes differentiation into parietal/visceral endoderm lineages. These data demonstrate that Nanog is essential for maintenance of a pluripotent status both *in vivo* and *in vitro*, and that *Nanog* function is to prevent endoderm differentiation (see Figure 6-7).

ES cells that express elevated levels of Nanog can self-renew in the absence of LIF, or even in the presence of LIF antagonists. These results indicate that *Nanog* is capable of rendering ES cells independent from LIF/STAT3 stimulation for self-renewal. However, *Nanog* and STAT3 exert their function independently from each other, as *Nanog* overexpression does not result in the activation of the STAT3 signaling pathway. In addition, Nanog overexpressing ES cells grown at clonal density in the presence of LIF and form increased numbers of purely undifferentiated colonies than do non-transgenic ES cells, indicating that LIF and *Nanog* have an additive effect on ES cell self-renewal. *Nanog* and *Oct4* work in concert to support stem cell potency and

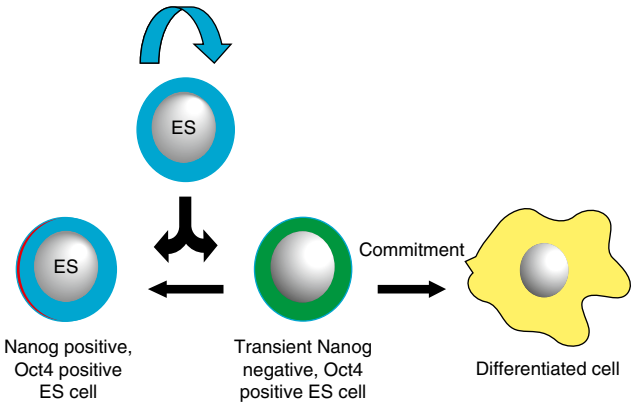


FIGURE 6-8 Nanog functions as a safeguard of pluripotency. A Nanog positive ES cell can either self-renew or give rise to a Nanog negative ES cell, which is predisposed towards differentiation while also being able to give rise to a Nanog positive ES cell. Adapted from Chambers *et al.*, 2007.

self-renewal. This conclusion is based on the two observations: first, *Nanog* is expressed in *Oct4*-deficient embryos; and second, *Nanog* overexpression cannot reverse the differentiation program induced in ES cells by *Oct4* down-regulation.

Chambers *et al.* further investigated the role of *Nanog* in ES self-renewal, and found that a fraction of ES cells that express *Oct4*, but do not have detectable *Nanog*, can still give rise to *Nanog*- and *Oct4*-positive cells upon trypsinization. Surprisingly, he found that permanent genetic ablation of *Nanog* does not abolish pluripotency, but rather reduces self-renewal capability by generating a transient state which is reversibly poised to both self-renewal and differentiation commitment (Figure 6-8). However, *Nanog*-deficient ES cells form PGCs that can colonize the genital ridge, but are unable to survive beyond E11.5. The conclusion of this study is that *Nanog in vivo* is essential for ICM determination and germ cell development. *In vitro* it works/acts by preventing progression to differentiation, and can thus be considered as a safeguard of pluripotency (Figure 6-8).

Nanog Regulation

Nanog proximal promoter (−289+117 relative to the ATG) contains a composite element of adjacent Sox and Oct binding sites, which are fully conserved among eutherian mammals. OCT4 and SOX2 control *Nanog* transcription by binding to this element in both mouse and human ES cells. The same Oct binding site was shown to mediate *Nanog* biphasic regulation by OCT4. Sub-steady state levels of *Oct4* expression elicit a positive effect on *Nanog* transcription, whereas steady state or higher doses exert a negative effect. This could explain why *Nanog* depletion and *Oct4* overexpression both lead to endoderm differentiation.

Nanog proximal promoter also mediates gene regulation via epigenetic mechanism. The −327 to −10 region is a tissue-dependent and differentially-methylated region or T-DMR. It contains six CpG dinucletides, which are found to be hypomethylated in ES cells and hypermethylated in trophoblastic stem and 3T3 cells.

Nanog and p53

Somatic cells respond to DNA damage by activating p53, which causes cell cycle arrest or apoptosis. However, ES cells lack p53-G1/S checkpoint and undergo apoptosis only in response to high levels of DNA damage. When low levels of DNA damage are introduced, ES cells tend instead to differentiate into a cell type, which is more prone to undergo p53-dependent cell cycle arrest and apoptosis. Two p53 binding motifs, located at −871 to −849 and −611 to −585 relative to the ATG, have been identified within the *Nanog* promoter region. P53 is known to interact with the corepressor mSin3a in order to repress transcription by recruiting histone deacetyleases. Upon DNA damage, p53 binds to *Nanog* promoter and suppresses Nanog transcription. P53 transcriptional activity and *Nanog* expression inversely correlate during RA-induced ES differentiation. However, p53 activity and *Nanog* suppression depend on p53 phosphorylated serine 315, which is essential for efficient recruitment of mSin3a to the Nanog promoter.

Nanog–Nanog Interaction

As NANOG is a homeobox potein, the possibility that it may homodimerize in order to exert target gene regulation was investigated. Co-immunoprecipitation experiments of tagged NANOG and deletion mutants of NANOG protein showed that NANOG can assemble into homodimers, and that dimerization is mediated by the tryptophan-rich C-terminal domain. NANOG homodimers are able to bind to *Nanog* consensus with high efficiency *in vitro*. Most importantly, Nanog dimers are responsible for directing ES cell LIF-independent self-renewal, as enforced expression of a Nanog mutant that does not contain the C-terminal domain leads to ES differentiation upon LIF removal.

PI3K and Nanog

Pharmacological inhibition of PI3K signaling with LY294002 results in reduction of *Nanog* mRNA and protein levels. In contrast, *Oct4* protein and messenger remain unaltered, indicating that the observed change in *Nanog* expression does not result from LY294002-induced global differentiation. Chemical inhibition of GSK3 activity reverses the effects of LY294002 treatment on *Nanog*. Since PI3K signaling leads to the AKT/PKB mediated inactivation of GSK3, these results suggest that the PI3K–GSK3 signaling axis is directly involved in the regulation of *Nanog* expression.

The *Oct4/FoxD3/Nanog* Regulatory Loop

FOXD3 was demonstrated to activate *Nanog* transcription in F9 EC cells through a binding site located between nt −544 and −269 of *Nanog* promoter. FOXD3 and NANOG in turn strongly activate a 2 Kb *Oct4* promoter, which interestingly is also repressed by *Oct4* itself. We mentioned earlier that OCT4 also acts as a biphasic regulator of *Nanog* expression. Since OCT4 concentration must be kept at precise levels in order to maintain stem cell pluripotency, these data point towards the existence of an *Oct4, Nanog*, and *FoxD3* regulatory circuit that would ensure the proper level of *Oct4* expression in ES cells.

THE CORE TRANSCRIPTIONAL REGULATORY CIRCUITRY CONTROLLING ES CELL IDENTITY

As mentioned before, in mouse ES cells expression of *utf1*, *fgf4*, *Rex1, Fbx15*, and *Nanog* is controlled by both SOX2 and OCT4. Remarkably, *Oct4* and *Sox2* genes are also regulated by the OCT4/SOX2 complex. OCT4 and SOX2 specifically mediate *Oct4* expression in ES cells by binding to site 2B of the Oct4 DE, whereas a non-canonical binding site for OCT4 and SOX2 within the 3′ Regulatory Region of the *Sox2* gene, called SRR2, is involved in *Sox2* expression in ES/EC cells. SOX2 and OCT4 or OCT6 can bind simultaneously, but not cooperatively, to SRR2. OCT6 or OCT4 (but not OCT1) have been shown to slightly increase *Sox2*-dependent transcription of the *Sox2* gene.

All these data suggest not only that *Oct4* and *Sox2* expression is auto-maintained, but also that pluripotency may be controlled by a restricted group of master regulators that could determine ES identity by regulating themselves together with crucial developmental factors. This hypothesis was confirmed to hold true both for mouse and human ES cells. Oct4/Sox2/Nanog binding sites along the human genome were mapped by "ChIP-on-chip" technology. The results of this genome-wide occupancy analysis revealed that *Oct4, Nanog*, and *Sox2* in the hES collaborate in forming a transcriptional regulatory circuitry consisting of both feedback autoregulatory and feed-forward loops. Remarkably, the three factors co-occupy a good portion of their target genes, with target co-occupancy leading to either target transcription activation or repression. ES cell-specific transcription factors and components of the Wnt/TGF-β signaling pathways belong to the group of genes that are activated by the OCT4/SOX2/NANOG, whereas development-related transcription factors like Neurogenin, Pax6, and Myf5 are among the negatively-regulated target genes. Oct4/Sox2/Nanog in hES team up as the main catalysts of ES identity by positively regulating signaling pathways that are essential to ES self-renewal, and by repressing genes required for developmental processes to occur. Genome-wide analysis of OCT4/NANOG binding sites also showed co-occupancy of the downstream target genes in mES cells. The tight functional association between OCT4 and NANOG is further supported by the discovery that the two proteins interact with each other. In addition, they associate with components (Mta1/2 and Hdca1/2) of the nuclear remodeling and histone deacetylation NurD and Sin3A complexes to co-occupy some of the known *Nanog* target genes, including *FoxD3*, *Oct4*, and *Nanog* itself.

Chromatin immunoprecipitation of the kruppel-like factors KLF2, KLF4, and KLF5 revealed that they bind and auto-regulate their own genes; they bind to *Nanog* enhancer and coregulate some downstream targets with NANOG. *Tcf3*, a terminal component of the Wnt canonical pathway, seems to be another integral component of the core regulatory circuitry of ES cells, as it was found to occupy, with OCT4 and NANOG, common ES and differentiation genes.

THE ROLE OF MICRO-RNAS IN PLURIPOTENCY

Small non-coding RNAs can be divided into two different classes: micro-RNA (miRNAs); and short interfering RNAs (siRNAs). Micro-RNAs control the stability and translation of target mRMAs and are involved in a wide variety of biological processes, including cell-cycle regulation, apoptosis, and cell differentiation. The double-stranded RNA-binding protein DGCR8 and the RNAse III enzyme Drosha process in the nucleus long primary miRNAs (pri-miRNAs) into short hairpins called precursor miRNAs (pre-miRNAs). The resulting hairpins are exported into the cytoplasm and processed by DICER into mature effector RNA molecules. DICER is also required for the maturation of short interfering RNAs, which regulate gene transcription by inducing cleavage of target mRNA.

Dicer

In vivo, Dicer is essential for mouse development and stem cell maintenance in the embryo. *Dicer*-deficient ES cells, which can be derived by gene targeting, express ES-cell specific markers but fail to differentiate both *in vivo* and *in vitro*. These ES cells also have high levels of double-stranded centromeric RNA transcripts that cannot be processed in the absence of *Dicer*. In addition, they display reduced levels of genomic DNA methylation and of histone H3-K9 di- and trimethylation at centromeric satellite repeats. These data indicate that *Dicer* is essential for stem cell differentiation, maintenance of centromeric heterochromatin structure and epigenetic centromeric silencing.

DGCR8

Significant reduction in the expression of the majority of known pre-miRNAs observed in *Dgcr8* knockout ES cells confirms that DGCR8 is essential for micro-RNA biogenesis. Although *dgcr8*-null ES cells fail to fully down-regulate pluripotency markers when induced to differentiate in the presence of retinoic acid, they do express some differentiation markers, albeit at lower levels than wild type cells.

This phenotype being different from the one described for *Dicer*-null ES cells, two conclusions can be drawn:

1. *Dicer* has miRNA-independent roles;
2. miRNA are essential for efficient silencing of the ES cell program during differentiation.

REST and miR-21

The neuronal repressor *Rest* is expressed at high levels in ES cells and down-regulated during differentiation. Both heterozygous deletion of *Rest* and its knockdown in ES cells cause a considerable loss of self-renewal and lead to the activation of markers specific for all lineages. Remarkably, decreased levels of *Oct4*, *Nanog*, *Sox2*, and *c-Myc* correspond to *Rest* transcript knocking down. Examination of miRNA profiles of *Rest*+/− and wt ES cells revealed a significant up-regulation of miR-21, -106a, -106b and -124a in heterozygous cells. However, only exogenous addition of precursor miR-21 to ES cells results in a decrease of self-renewal and in reduced expression of *Oct4, Nanog*, SOX2, and *c-Myc*. As REST binds to the miR−21 gene chromatin, it is proposed that *Rest* maintains self-renewal and pluripotency of mouse ES cells by suppressing miR−21 expression.

Notably, bioinformatics predicted miR−21 binding sites are present in *Sox2* and *Nanog* mRNAs. Additionally OCT4, NANOG, and SOX2 were found to co-occupy Rest promoter. REST was also found as part of the NANOG-OCT4 complex. All this data strongly point at Rest as a major component of the regulatory network regulating self-renewal and pluripotency of mouse ES cells.

THE CHROMATIN STATUS DEFINING STEM CELL IDENTITY

Embryonic stem cell identity depends on the combined expression of pluripotent genes and silencing of differentiation genes. However, both methylation of CpG dinucleotides and histone modifications have critical impact on transcription regulation by modulating global chromatin structure and accessibility. Therefore, gene profiling studies alone, while giving important information about the relative abundance of known transcription factors, are not sufficient to define cell identity. Thanks to the advent of ChIP-on-chip technology, a great amount of data resulting from genome-wide gene target, methylation, and chromatin analysis are now converging into the emergence of a global and comprehensive picture of stem cell configuration.

Histone H3 lysine 4 and lysine 27 methylation, respectively, mediate transcription activation and repression. Methylation of H3 lysine 4, which is catalyzed by the trithorax group of proteins (trxG), induces histone acetylation and nucleosome remodeling. Methylation of H3 lysine-27, which is catalyzed by the Polycomb Repressive Complex (PRC) PRC2, is required for PRC1-induced chromatin condensation, and consequently gene silencing. Polycomb group proteins (PcG) are the constituents of multiple PRC complexes and are conserved from Drosophila to humans. EZH2 is the methyltransferase component of the PRC2 complex.

Sequence analysis of 56 highly-conserved non-coding regions within the mouse genome showed significant enrichment for genes involved in embryonic development. So far, the distribution of repressive and activating histone modifications was thought to be non-overlapping. Surprisingly, genome-wide mapping of Histone H3 lysine 4 and 27 methylation within those 56 conserved non-coding regions revealed the existence of bivalent chromatin domains containing a combination of both repressive and active marks, i.e., H3 Lys4- and Lys27-methylation. Bivalent opposing marks are associated with low levels of expression in pluripotent cells, but these are resolved into a monovalent methylation mark during ES differentiation. Remarkably, genes with bivalent domains are mostly transcription factors, which are essential for differentiation to occur, hence the idea that the bivalent mark silences genes in ES cells but poises/primes them for activation upon differentiation. The significance of this new chromatin code, or signature of pluripotent cells, is further strengthened by the fact that similar bivalent marks were found in lineage-specific genes within the hES genome.

Another step forward in deciphering the chromatin code of pluripotency was taken with the studies conducted on the Polycomb group of proteins. The fact that PcG proteins may be important in dictating ES identity came from the observation that genetic ablation of *Suz12* and *ezh2*, both components of the PRC2 complex, results in early post-implantation lethality. Furthermore, ES lines cannot be derived from EZh2-deficient blastocysts

Mapping of SUZ12/RNA Pol II binding and of H3K27 trimethylation across the nonrepeat portion of hES genome by ChIP-on-chip analysis revealed that SUZ12 occupies and silences key genes involved in a variety of developmental processes (such as members of the Fox, Gata, and Tbx family). These developmental regulators are preferentially activated during differentiation, indicating that PcG-mediated silencing is associated with pluripotency and that loss of PcG occupancy correlates with differentiation (Figure 6-9). Similar results were obtained with mouse ES cells. However, a large number of genes, which are

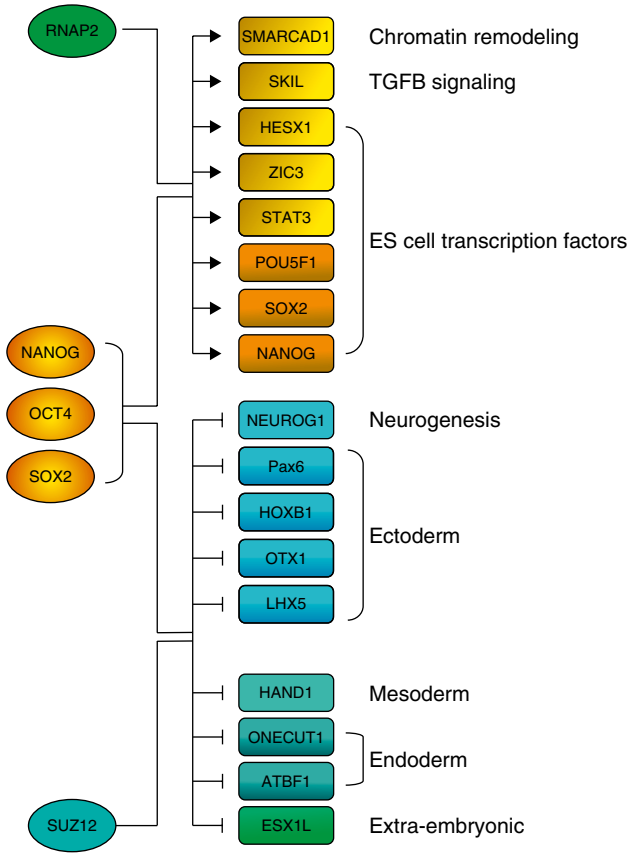


FIGURE 6-9 Examples of pluripotency associated genes co-occupied by Nanog/Oct4/Sox2 and RNAP2, and repressed developmental regulators co-occupied by Nanog/Oct4/sox2 and SUZ12 in hES cells. Adapted by Lee *et al*., 2006.

not marked by either histone modification or Polycomb protein association, are silenced in ES cells.

As previously stated, DNA methylation constitutes an important mechanism of gene expression regulation during embryogenesis. The two DNA methyltransferases *Dnmt3a* and 3b are essential for *de novo* methylation, and their ablation leads to early embryonic lethality. Triple mutant ES cells lacking *Dnmt3a*, 3b, and *Dnmt1* (a methyl DNA maintenance enzyme) can self-renew, but undergo massive apoptosis when stimulated to differentiate, indicating that DNA methylation likely contributes to the "pluripotent" configuration.

Genome-wide mapping of DNA methylation patterns at proximal promoters in mouse ES cells revealed that 87% of genes with neither H3K4me3 nor H3K27me3 are CpG methylated and mostly repressed, and that 71.3% of genes repressed by PcG complex are unmethylated. These results indicate that Polycomb association, histone modifications, and DNA methylation constitute distinct epigenetic regulators that work in a non-overlapping manner.

Importantly, a direct link exists between epigenetic control/chromatin modulation of ES cell identity and the pluripotency master gene *Oct4*. By ChIP analysis and transfection essays, OCT4 was found to bind and to activate the expression of Jmjd1a and Jmjd2c, which are respectively H3K9 Me2 and H3K9Me3 demethylases. *Tcl1* and *Nanog* are in turn downstream effectors of, respectively, Jmjd1a and Jmjd2c and specific knockdown of either one of the two demethylases leads to ES differentiation. Thus *Oct4*, *Tcl1*, and *Nanog* bridge the ES cell transcription circuitry to H3K9-mediated chromatin modulation.

REPROGRAMMING OF SOMATIC CELLS TO A PLURIPOTENT STATE: IPS VERSUS ES

Is pluripotency imposed by one single master gene or by a group of genes? Discovery of such a master gene(s) would constitute the panacea of modern human regenerative medicine, as it would obviate the need for human cloning, with all its ethical implications. From what was discussed in the previous paragraph, it appears that pluripotency may most likely be achieved through the combination of properly sequenced processes that modify chromatin accessibility or inaccessibility, and ultimately end with the activation and repression of pluripotency and differentiation genes, respectively.

Up to two years ago, reprogramming of a somatic cell to a pluripotent state could be accomplished only either by fusion with a pluripotent cell or by nuclear transfer into oocytes. In August 2006 Takahashi and Yamanaka demonstrated that pluripotent ES-like stem cells could be generated from both embryonic and adult mouse fibroblasts by retroviral transduction of four genes, i.e., *Oct4*, Sox2, Klf4, and c-Myc. The fibroblasts used in the study carried a β-geo knockin construct within the *Fbx15* alleles, which allowed isolation of reprogrammed fibroblasts by growth selection in G418. The induced pluripotent stem cells, named iPS, displayed morphology and growth properties typical of ES cells. Global gene-expression profiling of iPS revealed that these cells cluster more closely with ES cells than with fibroblasts. Although the microarray analysis showed genes commonly up-regulated in ES and iPS, it also highlighted genes which were expressed at higher levels in ES cells than in iPS. In addition, *Oct4* and *Nanog* promoters were found partially methylated in iPS cells, i.e., incompletely reprogrammed. When the pluripotent potential of the cells was tested, it turned out that, even though iPS were endowed with multi-lineage differentiation capability *in vitro*, *in vivo* they could contribute to fetal but not to adult mouse development. This was probably due to the fact that expression of all four factors was driven by constitutively active promoters, which are not able to mediate transgene down-regulation during differentiation. Altogether, these data indicated that *Fbx15*-iPS were similar, but not identical to ES cells.

iPS isolation from drug selected fibroblast carrying a neomycin gene inserted within the Oct4 or *Nanog* locus resulted instead in the generation of iPS with gene expression, chromatin, and DNA methylation characteristics, alias epigenetic state of pluripotent ES cells. Remarkably, *Nanog- and Oct4-neo* iPS displayed increased developmental potency, which was identical to the one showed by ES cells, as they were even germline competent. Expression analysis of transduced and endogenous *Oct4/Sox2/Klf4/c-Myc* genes showed that retroviral transgenes are silenced during the induction of *Oct4-* or *Nanog*-iPS. This result indicates that exogenous *Oct4, Sox2*, Klf4, and *c-Myc* are essential for the induction of ES-like characteristics, but that the activity of the endogenous genes must be engaged in order to achieve pluripotency and full differentiation potential. However, iPS-derived animals developed tumors, probably due to the reactivation of the *c-Myc* transgene. Successful reprogramming of fibroblasts was shown to be accomplished without *c-Myc*, but evidence that iPS derived by *Oct4*, *Sox2*, and *Klf4* transduction will not induce tumor formation in adult mice is still missing.

iPS can be isolated from non-transgenic fibroblasts by using exclusively morphological criteria for the recognition, and hence picking, of ES-like colonies after viral transduction. This method gives a rate of reprogramming efficiency that is 5–10-fold higher than that observed with the aid of drug selection. Importantly, fetal and adult fibroblasts are not the only differentiated somatic cell type that can give rise to iPS. Non-terminally differentiated B-lymphocytes were shown to be reprogrammed to a pluripotent state by inducible expression of the four factors, whereas mature B-cell reprogramming required additional genetic manipulation, leading to the suppression of the B-cell transcriptome, i.e., ectopic expression of the myeloid TF CCAAT/enhance-binding protein-alfa or knockdown of the B-cell specific transcription factor *Pax5*.

The derivation of iPS has finally brought into the stem cell field the sacred formula for the conversion of any cell into the ever young embryonic stem cell, but a mechanistic insight of the *in fieri* conversion is still fundamental for the understanding, and hence controlling, developmental potency. An attempt to fill up the gap came from two different studies. When the process of mouse iPS derivation was monitored at different time-points for pluripotency marker gene expression, alkaline phosphatase first, SSEA1, and finally *Nanog* and *Oct4* were consistently found to be switched on in a sequential temporal order. These data would point towards the existence of an *iter* of gene reprogramming which is defined and gradual, rather than chaotic. Further confirmation of this hypothesis was given by a comprehensive genomic/integrative genomic characterization of cells during iPS generation. Cells were transduced with inducible lentiviral vectors encoding for the four factors and sampled at day 4, 8, 12, and 16 of initial induction for expression profiling. The most immediate response to the four-factor induction consisted in the up-regulation of proliferation genes, probably mediated by *c-Myc*, and in the down-regulation of MEF-specific mesenchymal genes. Remarkably, a strong up-regulation of kidney neural epidermal lineage specific genes and of stress-induced antiproliferative genes (*Klf4* and *c-Myc* targets) was also observed, indicating that the majority of cells are prevented from reaching an embryonic status because they mostly tend to stop uncontrollable proliferation or/and activate a differentiation program which reflects *Sox2* and *Klf4* function in tissues other than the embryonic one. Fully reprogrammed cells express all known pluripotent genes and are epigenetically similar to ES cells, whereas partially reprogrammed cell lines, like the *Fbx15* iPS, reactivate only a subset of pluripotent genes, because of high levels of promoter methylation at pluripotent loci and incomplete down-regulation of lineage-specific genes. That DNA methylation status has a critical role in cellular reprogramming was also proved by the observed improvement in the reprogramming efficiency of fibroblasts treated with DNA methyltransferase inhibitors.

A further advancement in the understanding of the mechanism of pluripotency reacquisition occurred in July 2008 with the report of Kim and collegues. Neural stem cells (NSCs) express *Sox2* and *c-Myc* at levels that are about two-fold and ten-fold higher than in ES cells, respectively whereas *Klf4* expression is eight-fold lower. Exogenous Oct4, in combination with either *Klf4* or *c-Myc*, are sufficient to generate iPS cells from NSCs. These two-factor iPS are similar to ES cells at the molecular level, they form chimeras and are able to contribute to the developing germline. These data strongly support the hypothesis that the minimum number of factors required to fully revert somatic into pluripotent embryonic cells strictly depends on the level of expression of endogenous *Oct4, Sox2, Klf4*, and *c-Myc* relative to ES cells. Following the same hypothesis, if *Oct4* and *c-Myc* perform like *Oct4* and *Klf4*, then it is conceivable that even *Klf4* might be omissible in the reprogramming of NSCs, and that *Oct4* alone could fulfill the process. This was eventually found to be the case as Kim and co-workers succeeded in producing 1-factor iPS in February 2009, demonstrating that *Oct4* alone is required and sufficient to directly reprogram NSCs to pluripotency.

CONCLUSIONS

The therapeutic potential of induced pluripotent cells was tested in a sickle cell anemia mouse model. Autologous iPS were generated from skin cells of a diseased animal, targeted for correction of the endogenous human sickle hemoglobin gene and induced to differentiate into hematopoietic progenitors. The efficient treatment of sickle cell anemia obtained by transplantation of these host specific cells into the diseased mouse provides a proof of principle for the clinical achievements that combined gene and cell therapy can lead to. Importantly, induced pluripotent cells have also been derived from human fetal, neonatal, and adult fibroblasts by ectopic expression of the same four-factor cocktail that yielded mouse iPS. Unfortunately,

genetic manipulation of cells brings inevitable drawbacks: ectopic expression of *c-Myc* causes tumorigenicity, and random insertion of the viral sequences within the genome may produce unwanted mutagenesis events. Nonetheless, an essential step forward in coupled gene repair plus cell therapy has been made. We have learned that the fundamental transcriptional network governing pluripotency in humans and mice is conserved, regardless of the differences between the two species for growth factor requirements. As of today, the whole picture eventually appears in its marvelous completeness, thanks to Zhou and colleagues who apply the missing piece of the puzzle and obtained iPS by the 'simple' protein transduction of the 4 factors.

KEY TERMS

Pluripotent Able to give rise to differentiated derivatives of all three germ layers. Cells of the inner cell mass and ES cells are pluripotent.

Totipotent Able to give rise to all type of differentiated cells, including cells of the trophoectoderm lineage.

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Chapter 7

Stem Cell Niches

D. Leanne Jones and Margaret T. Fuller

INTRODUCTION

The ability of adult stem cells to both self-renew and produce daughter cells that initiate differentiation is the key to tissue homeostasis, providing a continuous supply of new cells to replace short-lived but highly differentiated cell types, such as blood, skin, and sperm. The critical decision between stem cell self-renewal and differentiation must be tightly controlled. If too many daughter cells initiate differentiation, the stem cell population may become depleted. Alternatively, unchecked stem cell self-renewal could abnormally expand the number of proliferative, partially-differentiated cells in which secondary mutations could arise, leading to tumorigenesis. A detailed understanding of how the choice between stem cell self-renewal and the onset of differentiation is determined may facilitate the expansion of adult stem cell populations in culture, while maintaining essential stem cell characteristics. This is a critical first step toward harnessing the potential of adult stem cells for tissue replacement and gene therapy.

STEM CELL NICHE HYPOTHESIS

Two classic developmental mechanisms can give rise to daughter cells that follow different fates. First, asymmetric partitioning of cell fate determinants in the mother cell can produce daughter cells that follow different cell fates, even though the two daughter cells reside in the same microenvironment. Second, the orientation of the plane of division can place two daughter cells in different microenvironments, which may then specify different cell fate choices through intercellular signaling. Ultimately, stem cell number, division, self-renewal, and differentiation are likely to be regulated by the integration of intrinsic factors and extrinsic cues provided by the surrounding microenvironment, now known as the "stem cell niche."

The concept of the stem cell niche arose from observations that many adult stem cells, such as hematopoietic stem cells, lose the potential for continued self-renewal when removed from their normal cellular environment, and the idea from developmental biology that different signaling microenvironments can direct daughter cells to adopt different fates. In the stem cell niche hypothesis, signals from the local microenvironment, or niche, specify stem cell self-renewal. If space within the niche were limited, such that only one daughter cell could remain in the niche, the other daughter cell would be placed outside of the niche, where it might initiate differentiation due to lack of self-renewal factors. However, if space within the niche is available or an adjacent niche is empty, both daughters of a stem cell division can retain stem cell identity. Therefore, the stem cell niche hypothesis predicts that the number of stem cells can be limited by the availability of niches with the necessary signals for self-renewal and survival. As a consequence, the niche provides a mechanism to control and limit stem cell numbers.

The existence of a stem cell niche has been proposed for several adult stem cell systems. The close proximity of the stem cells with respect to surrounding support cells plays an important role in the ability of the niche to adequately provide proliferative and anti-apoptotic signals, and to exclude factors that promote differentiation. Adhesion to support cells and/or a basement membrane holds stem cells close to such factors. In addition, the adhesion complexes themselves can provide polarity cues that influence orientation of stem cell divisions, so that one cell inherits the attachment and remains in the niche, while the other is displaced outside the niche into an alternate environment that encourages differentiation.

In this chapter, we review current knowledge of the role of the niche and the control of stem cell self-renewal using the *Drosophila* male and female germlines as model systems. We then briefly compare the molecular mechanisms revealed in the analysis of *Drosophila* germline stem cell niches to emerging pictures for how niches control stem cell behavior within other adult stem cell systems.

STEM CELL NICHES IN THE *DROSOPHILA* GERMLINE

The *Drosophila* germline has emerged as a valuable model system, providing significant insight into the regulation of

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stem cell behavior and the importance of the stem cell niche. Although in many systems, stem cells are rare and can be difficult to locate, the precise identity and location of the germline stem cells (GSCs) in the *Drosophila* ovary and testis are known. In addition, the availability of many mutants, a sequenced genome, and powerful genetic tools for cell type-specific ectopic expression has provided the opportunity to address essential questions regarding how stem cells interact with their surrounding microenvironment.

In *Drosophila*, the ability to generate clones of cells that are genetically distinct from neighboring cells allows both lineage tracing and analysis of the effects of lethal mutations during late stages of the life cycle, when lethality would already have occurred in an entirely mutant animal. Lineage tracing by analysis of marked clones has led to the identification of stem cells in both the male and the female germlines *in vivo*, within their normal environment. The genetically marked GSCs, as well as somatic stem cells, are identified by their ability both to be maintained and to continually produce a series of differentiating cells. Clonal analysis also allows the generation of mutant stem cells in an otherwise wild-type animal, allowing the analysis of a specific gene's function on stem cell maintenance, self-renewal, and survival.

In both the *Drosophila* ovary and testis, GSCs are in intimate contact with surrounding support cells that provide critical self-renewal and maintenance signals, thereby constituting a stem cell niche. Male and female GSCs normally exhibit oriented divisions and divide with invariant asymmetry, producing precisely one daughter stem cell that remains within the niche and one daughter cell that is displaced outside of the niche and will initiate differentiation.

Germline Stem Cell Niche in the *Drosophila* Ovary

The adult *Drosophila* ovary consists of approximately 15 ovarioles, each with a specialized structure, the germarium, at the most anterior tip (Figure 7-1A). Two to three GSCs

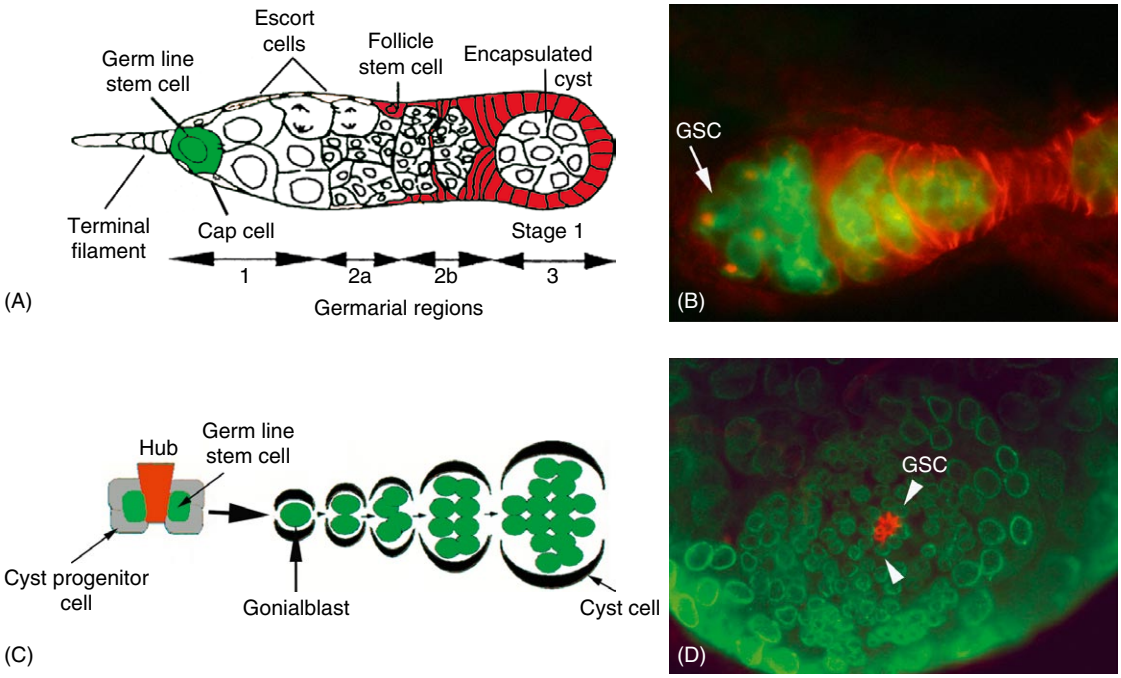


FIGURE 7-1 Germ-line stem cell niches in the *Drosophila* ovary and testis. (A) Schematic of a *Drosophila* germarium, which houses the germline stem cells (GSCs), anterior to the left and posterior to the right. The terminal filament, cap, and escort cells express molecules important for the maintenance and self-renewal of female GSCs, and comprise the stem cell niche. GSCs undergo asymmetric cell division, producing one daughter cell that will retain stem cell identity and one daughter cell, a cystoblast, that will initiate differentiation. As these divisions take place, the more mature cysts are displaced toward the posterior of the germarium. Cyst encapsulation by the follicle stem cell (FSC) derivatives occurs in region 2a–2b. Mature encapsulated cysts budding from the germarium make up region 3. (B) In the immunofluorescence image of a *Drosophila* germarium, germ cells are labeled with an antibody to the germ cell-specific protein, Vasa. Antibodies to the membrane protein α-spectrin label the somatic cells within the germarium, as well as a vesiculated, cytoplasmic ball-shaped structure known as the spectrosome in GSCs (arrow) and cystoblasts. (C) Schematic of the early steps in *Drosophila* spermatogenesis. GSCs surround and are in contact with a cluster of postmitotic, somatic cells known as the apical hub. The hub cells are a primary component of the male GSC niche. Each GSC is surrounded by two somatic stem cells, the cyst progenitor cells. The GSC undergoes asymmetric cell division, generating one daughter cell that will retain stem cell identity and one daughter cell, a gonialblast, which then undergoes four rounds of cell division with incomplete cytokinesis to produce 16 spermatogonia. The gonialblast is surrounded by cyst cells, which ensure spermatogonial differentiation. (D) In the immunofluorescence image of the apical tip of a *Drosophila* testis, the germ cells are labeled with an antibody to Vasa, and the somatic hub is labeled with an antibody to the membrane-associated protein, Fasciclin III. Eight GSCs (arrowheads) surround the apical hub.

lie at the anterior tip of the germarium, close to several groups of somatic cell types, including the terminal filament, cap cells, and escort cells (also known as inner germarial sheath cells) (Figure 7-1A, B). When a female GSC divides, the daughter cell that lies closer to the terminal filament and cap cells retains stem cell identity; the daughter cell that is displaced away from the cap cells initiates differentiation as a cystoblast. The cystoblast and its progeny undergo four rounds of cell division with incomplete cytokinesis. Of the 16 germ cells, only one will become the oocyte; the other 15 cells become nurse cells, which support the growth of the oocyte. The terminal filament, cap cells, and escort cells express molecules that regulate critical aspects of GSC behavior, constituting the germline stem cell niche in the ovary.

Xie and Spradling (2000) directly demonstrated the existence of a functional stem cell niche that can program cells to assume stem cell identity in the ovary. Taking advantage of a mutation that increases the rate at which stem cells are lost, Xie and Spradling showed that an empty stem cell niche is quickly filled by the division of a neighboring stem cell. In this case, the mitotic spindle of the GSC became reoriented parallel to the terminal filament and cap cells, and the stem cell divided symmetrically so that both daughters of the stem cell division became stem cells.

In the ovary, the vertebrate bone morphogenetic protein (BMP) 2/4 homolog Decapentaplegic (Dpp) is required for maintenance of GSCs. The cap cells and escort cells express *dpp*, which activates the Dpp signaling pathway in adjacent GSCs. Dpp binds and facilitates the association of type I and type II TGFβ class receptor serine/threonine kinases. As a result, the type II receptor phosphorylates and activates the type I receptor, which in turn phosphorylates the downstream mediator Mothers against Dpp (Mad). Mad facilitates nuclear translocation of Medea (Med), a transcriptional activator that stimulates Dpp target gene expression.

Excessive Dpp signaling can block germ cell differentiation in the *Drosophila* ovary. Overexpression of *dpp* results in enlarged germaria filled with cells resembling GSCs. TGFβ pathway signaling is also required for long-term maintenance of GSCs. Loss of function mutations in the type I receptor *saxophone* (*sax*) shortens the half-life of GSCs from one month to one week, and slows the rate of GSC divisions. Clonal analysis revealed that the downstream signaling components *mad* and *Med* are required cell autonomously in the germline for maintaining the normal half-life of GSCs. In the current working model, Dpp, which is secreted from supportive niche cells, signals to regulate the maintenance and rate of division of female GSCs. Further studies have demonstrated that Dpp signaling blocks differentiation by repressing transcription of the differentiation factor *bag of marbles* (*bam*) in GSCs.

The Piwi protein is also expressed in the terminal filament and cap cells and has been shown to act non-autonomously to support GSC maintenance in the *Drosophila* ovary. The *piwi* gene family, implicated in RNA silencing and translational regulation, plays crucial roles in stem cell maintenance in many organisms. In *Drosophila*, *piwi* mutant ovaries contain a normal number of primordial germ cells (PGCs) at the third instar larval stage, but adult ovaries contain only a few differentiating germ cells. Overexpression of *piwi* in the soma leads to an increase in GSCs in the germarium, suggesting a role in GSC self-renewal. Piwi is also expressed in the germline, where it appears to play a cell-autonomous role in controlling the rate of GSC division in the ovary. Null mutations in *piwi* also result in a failure to maintain *Drosophila* male GSCs, although the mechanism by which Piwi acts on GSCs in the male has not been established.

Germline Stem Cell Niche in the *Drosophila* Testis

The *Drosophila* adult testis is a long, coiled tube filled with cells at all stages of spermatogenesis. In adult *Drosophila melanogaster*, approximately nine GSCs lie at the apical tip of the testis, forming a ring that closely surrounds a cluster of post-mitotic somatic cells called the hub (Figure 7-1C, D). When a male GSC divides, it normally produces one cell which will retain stem cell identity, and one cell, called a gonialblast, that is displaced away from the hub and will initiate differentiation (Figure 7-1C). The gonialblast and its progeny undergo four rounds of transit-amplifying mitotic divisions with incomplete cytokinesis, creating a cluster of 16 interconnected spermatogonia.

In the *Drosophila* testis, signaling through the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway has been shown to regulate the self-renewal of male GSCs. Hub cells express the ligand Unpaired (Upd), which activates the JAK-STAT pathway in the adjacent stem cells and specifies stem cell self-renewal. *Drosophila melanogaster* has one known JAK, encoded by the *hopscotch* (*hop*) gene, and one known STAT, Stat92E. In males carrying a viable, male sterile *hop* allele, the initial round of germ cell differentiation occurs, but GSCs are lost soon after the first rounds of definitive stem cell divisions at the onset of spermatogenesis. Clonal analysis of homozygous mutant germ cells demonstrated that *Stat92E* activity is required cell autonomously in the germline for stem cell maintenance. Upd is normally expressed exclusively in the hub cells; however, ectopic expression of *upd* in early germ cells resulted in an enlarged testis tip filled with thousands of small cells resembling GSCs, gonialblasts, and early somatic cyst cells. Together, these data suggest that the somatic apical hub cells act as a major component of the GSC niche in the testis by providing a source of Upd. Experiments in tissue culture suggest that

Upd protein associates with the extracellular matrix upon secretion. If binding to the extracellular matrix restricts Upd diffusion *in vivo*, then only cells that maintain direct contact with the hub may receive sufficient levels of Upd to retain stem cell identity. Consistent with this hypothesis, activation of Stat92E, the sole *Drosophila* STAT homolog, is observed only in hub cells and the adjacent stem cells.

Coordinate Control of Germline Stem Cell and Somatic Stem Cell Maintenance and Proliferation

Multiple stem cell populations can reside within a common anatomical location—for example, hematopoietic and mesenchymal stem cells in the bone marrow. Coordinated control of the proliferation of different stem cell types may be especially important when the two stem cell types generate differentiated cell populations that must work together to maintain a tissue. The *Drosophila* female and male gonads provide excellent systems in which to study how the behavior of two such stem cell populations, somatic and germline, can be controlled coordinately.

In the *Drosophila* ovary, the germarium houses two populations of stem cells in addition to GSCs. Escort stem cells (ESCs) are the source of escort cells (ECs) that encapsulate each cyst of interconnected germ cells in regions 1/2A. Subsequently, follicle stem cells (FSCs) produce the many specialized follicle cells that cover each developing egg chamber. Lineage tracing, achieved through clonal analysis, demonstrated that FSCs are located at the border between regions 2A–2B, several cell diameters from the female GSCs in the ovariole (Figures 7-1A, B).

The JAK-STAT pathway has also been implicated in the proliferation and maintenance of the ESCs. A reduction in STAT function led to loss of escort cells and GSCs, while overexpression of *upd* in somatic cells led to an accumulation of both germ cell cysts and escort cells. Subsequent clonal analysis revealed a specific requirement for STAT in ESCs for proper ESC function, maintenance, and germarium structure. In contrast, the Hedgehog (Hh) signal transduction pathway has been implicated in controlling the proliferation and differentiation of the FSCs and their progeny. Hh is strongly expressed in terminal filament and cap cells at the tip of the germarium (Figure 7-1A). Loss of *hh* activity reduced the number of somatic cells in the germarium. Consequently, fewer follicle cells are available to intercalate between adjacent germline cysts, resulting in an accumulation of unencapsulated cysts in the germarium. Overexpression of *hh* in the ovary leads to hyperproliferation of somatic cells, resulting in increased numbers of cells that separate adjacent egg chambers and increased numbers of specialized follicle cells at the poles of developing egg chambers. At this time, it is not clear how *hh*, expressed in the terminal filament and cap cells, might regulate FSC proliferation, as the FSCs lie several cell diameters away. It is possible that the FSCs receive the Hh signal directly, or that Hh is also signaling through some other somatic cell type, escort cells for example, to control the proliferation of FSCs indirectly (Figure 7-1A).

The *fs(1)Yb* gene may serve as a coordinate upstream regulator of both GSC and SSC proliferation in the *Drosophila* ovary by controlling the expression of both *piwi* and *hh* within the niche. Yb protein is expressed in the terminal filament and cap cells, and *Yb* mutants exhibit reduced expression of Hh and Piwi protein in cap cells and somewhat reduced expression in terminal filament cells. Loss of function of *Yb* results in loss of GSCs, similar to that observed in *piwi* mutants. In both cases there is precocious differentiation of GSCs without apparent self-renewal, and a concomitant reduction in the number of somatic cells. Consequently, ovarioles consist of several differentiating germline cysts and a germarium devoid of germ cells. Conversely, overexpression of *Yb* leads to phenotypes similar to those seen on ectopic expression of *piwi* and *Hh*: increased numbers of both GSCs and somatic cells in the germarium.

In the *Drosophila* testis, somatic cyst stem cells (CySC) flank the male GSCs and directly contact the apical hub cells using thin cytoplasmic extensions (Figure 7-1C). CySC self-renew and generate daughter cells that differentiate into somatic cyst cells. They can also generate daughter cells that contribute to the hub. Cyst cells in the testis appear to be analogous to escort cells at the anterior tip of the germarium. Two somatic cyst cells, which may be the functional equivalent of mammalian Sertoli cells, enclose each gonialblast and its progeny, and play a major role in ensuring spermatogonial differentiation (Figure 7-1C).

The number of early somatic cells at the testis tip decreases dramatically in *hop* mutant testes. Reciprocally, the number of early somatic cells increases in response to ectopic expression of Upd in early germ cells, suggesting that Upd secreted from the apical hub signals directly to the somatic, as well as to the GSC, populations to specify stem cell self-renewal. Interestingly, Leatherman and DiNardo (2008) recently showed that overexpression of an activated form of Hop within the somatic cyst cells, but not germ cells, results in overproliferation of both somatic and germline stem cell populations, indicating that cells in the somatic cyst lineage non-autonomously regulate GSC proliferation. These recent data highlight the complexity of signaling within stem cell niches and the cooperative relationship between stem cells residing within the same niche.

Structural Components of the Niche

In *Drosophila* gonads, adhesion between GSCs and the surrounding support cells is important for holding stem cells

within the niche, close to self-renewal signals and away from differentiation cues. In addition, clustering of adherens junctions along the stem cell surface may impart polarity to the stem cell to ensure asymmetric divisions. GSCs in the ovary and testis appear to make direct cell–cell contact with surrounding support cells. Clusters of adherens junctions are observed between female GSCs and cap cells, as well as between male GSCs and the adjacent hub cells. Immunofluorescence analysis revealed that the *Drosophila* E-cadherin homolog Shotgun (Shg) and β-catenin homolog Armadillo (Arm) are highly concentrated at the interface between the GSCs and the cap cells in females, and between the GSCs and the hub cells in the male. Recruitment and maintenance of female GSCs within the niche requires the activity of both *shg* and *arm* (Song *et al.*, 2002). Removal of *shg* activity from the germline using clonal analysis resulted in failure of female GSCs to be efficiently recruited into their niches in the developing ovary. Furthermore, *shg* mutant GSCs that were recruited to the niche were not maintained, suggesting that *D*E-cadherin-mediated cell adhesion is required for holding GSCs in their niche in the germarium. This in turn is required for efficient stem cell self-renewal. Voog *et al.* (2008) recently demonstrated a similar requirement for *shg* in the maintenance of both GSCs and CySCs in the *Drosophila* testis. However, it is also clear that in some cases, such as for intestinal stem cells in the *Drosophila* midgut or satellite cells in mammalian muscle fibers, adhesion to a basement membrane may serve as the primary mechanism for anchoring stem cells within the niche.

Gap junctional intercellular communication via transfer of small molecules may also be involved in the survival and differentiation of early germ cells in the *Drosophila* ovary. Mutations in the *zero population growth* (*zpg*) gene, which encodes a germline-specific gap junction protein, result in loss of early germ cells at the beginning of differentiation in both males and females. Zpg protein is concentrated on the germ cell–soma interface in males and females, and between adjacent germ cells in developing egg chambers. Transfer of small molecules, nutrients, or both from surrounding support cells to germ cells via gap junctions may be essential for the survival of early germ cells undergoing differentiation. The presence of gap junctions between female GSCs and adjacent support cells, coupled with the eventual loss of GSCs in *zpg* mutants, also suggests that signaling via gap junctions may play a role in stem cell maintenance or may help physically maintain GSCs in their niche.

In summary, the male and female germlines of *Drosophila* have provided genetic systems in which to test the principles and investigate the basic underlying mechanisms of the stem cell niche theory. Clonal marking experiments conclusively identified GSCs *in situ* in both the testis and ovary, allowing the study of the relationship between these stem cells and their surrounding microenvironment. Several themes arising from the analysis of *Drosophila* male and female GSCs offered potential paradigms for analysis of stem cell niches in mammalian systems. First, stem cells are usually located adjacent to support cells that secrete factors required for maintaining stem cell identity: the hub cells at the apical tip of the testis and the cap cells in the germarium at the tip of the ovary. The signal transduction pathways involved in stem cell maintenance may not be conserved between male and female GSC systems, nor are they necessarily conserved between GSC and somatic stem cell populations within the gonads. Second, cell–cell adhesion between GSCs and niche cells may be required for stem cell maintenance, physically maintaining stem cells within the niche and ensuring that GSCs are held close to self-renewal signals emanating from the microenvironment. Third, two or more stem cell types can be maintained within the same niche in an anatomical arrangement that facilitates the interaction of cell daughters from disparate origins that, in some cases, may undergo coordinated differentiation.

STEM CELL NICHES WITHIN MAMMALIAN TISSUES

Specialized niches have been proposed to regulate the behavior of stem cells in several mammalian tissues maintained by stem cell populations, including the male germline, the hematopoietic system, the epidermis, and the intestinal epithelium. Many of these niches share several characteristics with stem cell niches in the *Drosophila* germline, specifically signaling molecules secreted from the surrounding microenvironment and cell adhesion molecules that anchor stem cells within the niche.

Mammalian Testis

The seminiferous tubules of the mammalian testis are the site of ongoing spermatogenesis in the adult. In the embryo, primordial germ cells (PGCs) divide and migrate to the genital ridges. In the developing seminiferous cords, the PGCs become gonocytes, which home to the basement membrane of the seminiferous tubules, where they differentiate into spermatogonial stem cells (SSCs) and initiate self-renewing divisions. The A_S (single) spermatogonia, the presumptive stem cells, are found close to several groups of supporting somatic cells, including the peritubular myoid and Sertoli cells, which may contribute to the stem cell niche (Figure 7-2). In addition, recent data using Neurogenin 3 as a marker for undifferentiated spermatogonia has demonstrated that A_{undiff} spermatogonia, including A_S cells, are not randomly located along the periphery of the seminiferous tubules. Rather, these cells appear to cluster adjacent to the vasculature and interstitial cells between adjacent tubules, suggesting that vasculature and/or interstitial cells may significantly

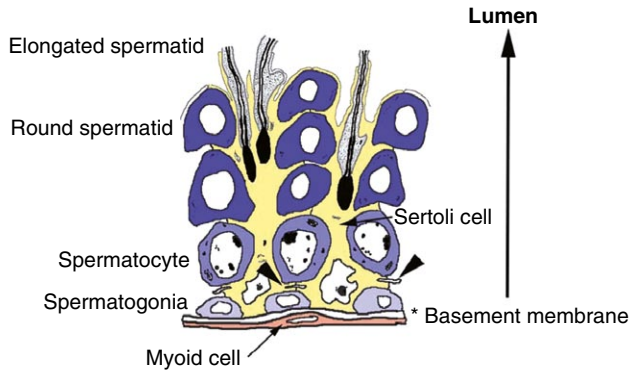


FIGURE 7-2 Organization of germ cells and somatic cells within a seminiferous tubule. Spermatogenesis occurs inside the seminiferous tubules that make up the testis. The mammalian male germline stem cells (A_s spermatogonia) lie at the periphery of the seminiferous tubules adjacent to the basement membrane (asterisk), and differentiation proceeds through multiple stages, creating spermatogonia, spermatocytes, spermatids, and spermatozoa, which are released into the lumen of the tubule. The spermatogonia are in close association with several somatic cell types, including the peritubular myoid cells and Sertoli cells. Sertoli cells flank germ cells of all stages and are joined continuously around the tubule by tight junctions (arrowheads). Sertoli cells and myoid cells are strong candidates for cellular components of the stem cell niche within the testis.

contribute to the stem cell niche within the mammalian testis (Yoshida *et al.,* 2007).

SSCs taken from a fertile mouse or rat can be transplanted into the seminiferous tubules of an immunodeficient mouse recipient. These exogenous stem cells are able to migrate through layers of differentiating germ cells and Sertoli cell tight junctions to find the stem cell niche along the basement membrane and establish colonies of donor-derived spermatogenesis. Transplantation experiments have demonstrated that both the number of stem cells and the available niches increase with age and testis growth further more the microenvironments within testes from immature pups were better at allowing colonization events, whether the donor stem cell was from an adult or from a pup.

Secreted signaling molecules that infulence the proliferation of mammalian SSCs have not yet been identified. Sertoli cells produce a growth factor, glial cell line-derived neurotrophic factor (GDNF), which affects the proliferation of premiotic germ cells, including stem cells. Depletion of stem cell reserves is observed in mice lacking one copy of GDNF. Conversely, mice overexpressing GDNF under the control of a promoter that drives preferential expression in the germline show accumulation of undifferentiated spermatogonia that neither differentiate nor undergo apoptosis. Older GDNF-overexpressing mice regularly form nonmetastatic testicular tumors, thus suggesting that GDNF contributes to paracrine regulation of spermatogonial proliferation and differentiation.

The development of a transplantation assay to test the function of mammalian SSCs has provided a framework on which to begin the molecular characterization of spermatogonial stem cells and of the stem cell niche in the adult mammalian testis.

Enrichment of stem cells using FACS sorting and monoclonal antibodies for specific surface markers, followed by transplantation of sorted populations, has led to identification of α6 integrin as a candidate surface marker for spermatogonial stem cells, raising the possibility that attachment of stem cells to the ECM may be important for stem cell maintenance. The association of spermatogonia and differentiating spermatocytes with Sertoli cells is likely to be mediated in part by adherens junctions, although the precise cadherin and cadherin-like molecules involved in this cell–cell interaction have not been conclusively identified. Meanwhile, Sertoli cells lining the tubules are continuously joined by tight junctions that regulate the movement of cells and large molecules between the basal compartment and the lumen of the seminiferous tubules (Figure 7-2).

Hematopoietic System

The major anatomical sites of hematopoiesis change during ontogeny. Hematopoietic stem cells (HSCs) are first present in the embryonic yolk sac and the aorta–gonad–mesonephros (AGM) region, followed by transition to the placenta, the fetal liver and spleen. Just before birth, HSCs migrate to the bone marrow, where blood formation is maintained throughout the lifetime of the animal.

HSCs reside along the inner surface of trabecular bone, and differentiating cells migrate toward the center of the bone marrow cavity. The bone-forming osteoblasts have been proposed to be a major cellular component of the HSC niche, as increases in the number of osteoblasts led to a concomitant increase in the number of long-term HSCs. These osteoblasts secrete an elevated level of the Notch ligand Jagged-1, and Notch signaling has been implicated in regulating the fate of HSCs by blocking differentiation. HSCs also localize adjacent to vascular endothelial cells, which also contribute to the HSC niche. Recent results suggest that the pool of HSCs is quite heterogeneous with respect to proliferative potential, and that there may be two populations of HSCs with long-term reconstitution potential: "dormant" HSCs (dHSCs) and more actively proliferating HSCs (aHSCs). The existence of multiple pools of stem cells could provide another mechanism to ensure sufficient stem cells for ongoing tissue maintenance as well as a reservoir for repair after injury. It will be interesting to see whether the two HSC states correlate with localization next to trabecular osteoblasts versus vascular endothelial cells.

Interestingly, HSCs are mobile and detectable in the peripheral blood, spleen, and liver, and localization adjacent to vasculature probably facilitates HSC migration into and out of the niche. The mobility of HSCs suggests that

adhesion between HSCs and supportive niche cells is highly regulated, and although specific cadherins and integrins have not been identified, adhesion molecules such as membrane-bound Steel factor and CXCR4 appear to play a role in holding HSCs within the niche. The mechanisms that recruit HSCs back into the niche after migration or homing of HSCs to the bone marrow after transplantation have not been clearly elucidated, although cellular adhesion molecules and chemokine receptors are likely involved.

Studies have shown that signaling through the canonical Wnt pathway is also important for regulating HSC fate by specifying stem cell self-renewal. Wnt is a secreted growth factor that binds to members of the Frizzled (Fz) family of cell surface receptors. The β-catenin molecule serves as a positive regulator of the pathway by mediating transcription, in cooperation with members of the Lef–TCF transcription factor family. In the absence of a Wnt signal, cytoplasmic β-catenin is quickly degraded through the ubiquitin–proteasome pathway. Transduction of HSCs with a retrovirus encoding a constitutively active β-catenin molecule resulted in self-renewal and expansion of HSCs in culture for at least four weeks, and in some cases as long as one to two months, under conditions in which control HSCs did not survive in culture beyond 48 hours. The cultured cells resembled HSCs morphologically and phenotypically, and were capable of reconstituting the entire hematopoietic system of lethally irradiated mice when transplanted in limiting numbers. Proliferation of wild-type HSCs cultured in the presence of growth factors was blocked by a soluble form of the ligand-binding domain of the Fz receptor, suggesting that Wnt signaling is required for the proliferation response of HSCs to cytokines within their niche. Because no other cell types were present in these cultures, this result raises the possibility that a Wnt secreted from HSCs may act as an autocrine signal to promote HSC self-renewal and maintenance.

Mammalian Epidermis

The mammalian epidermis is comprised primarily of keratinocytes, a sub-population of which is stem cells. Epidermal stem cells within the hair follicle are multipotential; they produce progeny that differentiate into interfollicular epidermis and sebocytes, and contribute to all the differentiated cell types involved in forming the hair follicle, including the outer root sheath, inner root sheath, and hair shaft.

It is not clear whether one "primordial" epidermal stem cell creates the stem–progenitor cell populations that maintain the interfollicular epidermis, the hair follicle, and sebaceous gland, or whether the stem cells that maintain each of these specific cell types are equivalent, with their fate determined by the local environment. However, accumulating evidence supports a model whereby the microenvironment, or niche, affects differentiation toward particular lineages. For example, cultured rat dermal papillae cells can induce hair follicle formation by rat footpad epidermis, in which follicles are not normally found. These data suggest that stem cells that normally maintain the interfollicular epidermis, called basal keratinocytes, can be reprogrammed to act as hair follicle stem cells by signals emanating from the surrounding microenvironment. For this chapter, we consider the stem cells that generate the hair follicle and the interfollicular epidermis separately.

Hair Follicle

After placement and formation of the hair placode during mammalian embryonic development, the lower portion of the hair follicle cycles through periods of growth (anagen), regression (catagen), and quiescence (telogen). The proliferative cells that generate the inner root sheath and hair shaft are called matrix cells, a transiently dividing population of epithelial cells at the base of the hair follicle that engulfs a pocket of specialized mesenchymal cells, called the dermal papilla (Figure 7-3A).

By using multiple strategies, a stem cell niche for the mammalian epidermis has been located along the upper portion of the hair follicle in a region at or just under the bulge. The bulge is located along the outer root sheath, which is contiguous with the interfollicular epidermis (Figure 7-3A). As the hair follicle regresses during catagen, the dermal papilla comes into close proximity with the follicular bulge. It has been suggested that one or more signals from the dermal papilla may cause stem cells, transit-amplifying cells, or both to migrate out and begin proliferating to regenerate the hair follicle.

Both in human and in mouse epidermis, β1 integrin expression is enriched in cells within the bulge region of the outer root sheath. Targeted disruption of the β1 integrin gene in the outer root sheath cells did not disrupt the first hair cycle. However, proliferation of matrix cells was severely impaired, resulting in progressive hair loss and dramatic hair follicle abnormalities. Proliferation of basal keratinocytes was also significantly reduced, and by seven weeks, these mice completely lacked hair follicles and sebaceous glands, suggesting that β1 integrin function is required for normal epidermal proliferation. It is possible that one role of β1 integrin is to anchor stem cells in or near the bulge, close to self-renewal signals.

Both the Sonic hedgehog (Shh) and the Wnt/β-catenin signaling pathways have been shown to affect some aspects of cell proliferation and differentiation in the epidermis and epidermal appendages. In addition, BMP signaling appears to maintain the quiescence of follicle stem cells, rather than the stem cells, *per se*. Shh signaling appears to specify hair follicle placement and growth during embryogenesis, as well as postnatal follicle regeneration. Shh is expressed at the distal portion of the growing hair follicle, along one

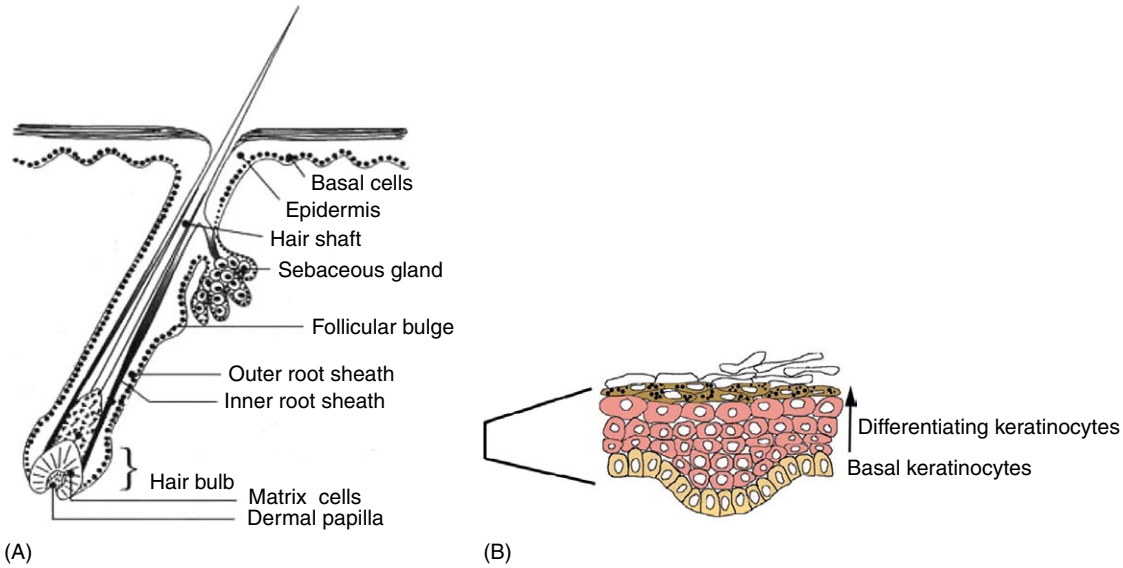


FIGURE 7-3 (A) Schematic of the components of a hair follicle (modified from Watt, 2001). The follicular bulge (asterisk) has been proposed to act as a stem cell niche, which houses cells that can contribute to all the differentiated cell types involved in the formation of the hair follicle, including the outer root sheath, inner root sheath, and hair shaft. Stem cells within the bulge can also generate sebocytes and the cells that maintain the interfollicular epidermis. The bulge is located along the outer root sheath, which is contiguous with the interfollicular epidermis (dotted line). (B) Drawing of a cross-section of the interfollicular epidermis. The stem cells that maintain the interfollicular epidermis are within the basal layer of the epidermis and divide to produce the transit-amplifying cells, which undergo a process of terminal differentiation as they migrate toward the surface of the skin. Dead squamae are shed from the surface of the skin. Interfollicular epidermal stem cells are found in patches, surrounded by transit-amplifying cells that form an interconnecting network between the stem cell clusters.

side of the matrix closest to the skin surface (Figure 7-3A). Interestingly, fibroblast growth factors (FGFs) and BMPs, expressed in the dermal papilla, affect hair follicle growth by regulating *Shh* expression in matrix cells (Figure 7-3A).

Loss of *Shh* function leads to disruption in hair follicle growth, while ectopic expression of Shh target genes induces follicular tumors. In addition, basal cell carcinomas, caused by mutations in downstream components of the Shh pathway, are composed of cells similar to hair follicle precursor cells. Shh expression in the epithelium results in the expression of target genes such as *Patched* (*Ptc*) in both the proliferating matrix cells and the adjacent dermal papilla. Because Shh targets are expressed in both the epidermis and the underlying dermal tissue, it is unclear whether the effect of Shh on epithelial cell proliferation is direct or indirect.

Wnt signaling also plays an important role in the formation of hair follicles during embryogenesis and postnatal specification of matrix-derived cells into follicular keratinocytes. Overexpression of a stabilized form of β-catenin in murine skin leads to the formation of ectopic hair follicles and hair follicle-derived tumors. Alternatively, a skin-specific knockout of β-catenin attenuated hair germ formation during embryogenesis, and dramatically restricted specification of cell fates by the multipotent bulge stem cells after completion of the initial hair cycle. At the initiation of the second growth phase, β-catenin-deficient stem cells were incapable of differentiating into follicular epithelial cells, and were restricted to producing interfollicular keratinocytes.

Several members of the Lef1–Tcf family, as well as numerous Wnts, are expressed in the skin. Cells within the bulge and the lower outer root sheath express *Tcf3*;*Lef1* is highly-expressed in the proliferative matrix cells and differentiating hair-shaft precursor cells. Experiments suggest that Tcf3 acts as a repressor to maintain follicle stem cells in an undifferentiated state, and may do so independently of binding to β-catenin. Tcf3-repressed genes include transcriptional regulators of the epidermal, sebaceous gland, and hair follicle differentiation programs. Upon Wnt signaling, cells initiate differentiation along the hair follicle lineage. Lef1, on the other hand, requires binding to β-catenin, and presumably activation by one or more Wnts to mediate its effects on hair follicle differentiation. Although these data re-emphasize a role for Wnt signaling in hair follicle generation, there is still no evidence that Wnts directly control the proliferation, maintenance, or self-renewal of epidermal stem cells.

Stabilization of β-catenin through Wnt signaling, in concert with the activation of *Lef1* transcription by repression of BMP signaling by noggin, acted to repress E-cadherin expression and to drive follicle morphogenesis. Conditional removal of α-catenin also resulted in the arrest of hair follicle formation, and the subsequent failure of sebaceous gland formation. Together, these results highlight the importance of Wnt signaling, and of the regulation of adherens

junction formation in the development and maintenance of hair follicles.

Interfollicular Epidermis

Stem cells in the bulge can migrate superficially to maintain the interfollicular epidermis. The stem cells that maintain the interfollicular epidermis, basal keratinocytes, reside within pockets in the basal layer of the epidermis and divide to generate the transit-amplifying cells, which undergo a process of terminal differentiation as they migrate toward the surface of the skin (Figure 7-3B). Basal keratinocytes are found in patches, surrounded by transit-amplifying cells that form an interconnecting network between the stem cell clusters.

Adhesion to the extracellular matrix promotes stem cell identity and prevents differentiation of keratinocytes. Human or mouse basal keratinocytes can be grown and cultured *in vitro*, and cultured adult human keratinocytes have been used as autografts in the treatment of burn victims for the past 20 years. The ability to culture keratinocytes *in vitro* has also allowed the development of potential strategies to use cutaneous gene therapy to correct various skin disorders and chronic wounds.

When cultured keratinocytes are placed in suspension, they immediately cease cell division and initiate differentiation. Keratinocytes express a variety of integrins, and although some are generally expressed, others are induced only during development, wounding, and disease. All cells within the basal layer of the epidermis express β1 integrin. However, the cells most likely to be stem cells within the interfollicular epidermis express surface levels of β1 integrin two- to threefold higher than those of transit-amplifying cells. High levels of β1 integrin with signaling through the mitogen-activated protein kinase cascade were demonstrated to promote stem cell identity in basal keratinocytes. As a result, a high level of β1 integrin is commonly used as a visual marker for stem cells within the interfollicular epidermis.

Activated β-catenin and signaling through Tcf has been demonstrated to increase the proportion of stem cells in keratinocyte cultures. Cultured mouse keratinocytes exposed to Wnt3a- and noggin-conditioned media showed a significant increase in β-catenin and Lef1 levels, and in localization of Lef1 to the nucleus. These two signaling pathways could act to reduce *E-cadherin* expression in interfollicular basal keratinocytes, similar to the manner in which they act to regulate *E-cadherin* expression during hair follicle morphogenesis. Interestingly, stem cells in the interfollicular epidermis have surface levels of E-cadherin lower than those of transit-amplifying cells.

Notch signaling has been suggested to promote differentiation of interfollicular keratinocytes. High levels of the Notch ligand, Delta1, in cultured human epidermal keratinocytes signal to adjacent cells to differentiate, while the stem cells are protected from this signal. Notch signaling has also been demonstrated to stimulate differentiation in mouse epidermal cells. Accordingly, conditional removal of *Notch* from basal keratinocytes results in epidermal hyperplasia, suggesting that Notch negatively regulates epithelial stem cell proliferation, and may act as a tumor suppressor in mouse epidermis. Interestingly, *Notch1* deficiency also results in increased levels of β-catenin and Lef1 and in the formation of basal cell carcinoma-like tumors.

Gut Epithelium

The inner lining of the colon and small intestine is a simple columnar epithelium, constantly renewed by the proliferation of stem cells residing within pockets, or crypts, along the intestinal wall. Intestinal cells leave the crypt at a rate of 200–300 cells/day and migrate onto ciliated villi that protrude into the gut lumen (reviewed by Winton, 2001; Figure 7-4A). Recently, Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5, also known as Gpr49) was characterized as a marker that allows identification and characterization of intestinal stem cells, called crypt-base columnar cells (CBCs), in both the small intestine and colon. Lineage-tracing experiments demonstrated that the presumptive stem cells of both the small intestine and the colon reside near the base of each crypt, with the CBCs in the small intestine interspersed among the Paneth cells. Within a crypt, approximately four to five stem cells generate transit-amplifying cells, which are capable of up to six transit divisions. Migration of these transit-amplifying cells out of the proliferative zone is required for the onset of differentiation (Figure 7-4B). The stem cells may be maintained at the base of crypts, embedded in the intestinal wall, for protection from toxins passing through the gut lumen.

Wnt signaling is involved in controlling the proliferation and differentiation of intestinal epithelial cells. In fact, Lgr5 was initially characterized as a Wnt target gene. In humans, mutations in the adenomatous polyposis coli (*APC*) gene, a negative regulator of Wnt signaling, are etiologically linked to the development of colorectal cancers. Also, constitutively active nuclear complexes of Tcf4–β-catenin are found in *APC* −/− colon carcinoma cell lines, or in cell lines that have a stable form of β-catenin, suggesting that hyperactivation of Tcf4 may contribute to cellular transformation. Loss of the Tcf4 transcription factor, which is expressed in the intestinal epithelium, leads to the depletion of stem cells and failure to maintain the proliferative compartments in the intervillus pockets of the neonatal small intestine. This phenotype was not evident in the colon, suggesting that another Tcf family member may act redundantly with, or instead of, Tcf4 in the large intestine.

If crypts serve as a niche to support the self-renewal of intestinal stem cells, this implies that cells near the intestinal stem cells may be the source of a secreted self-renewal

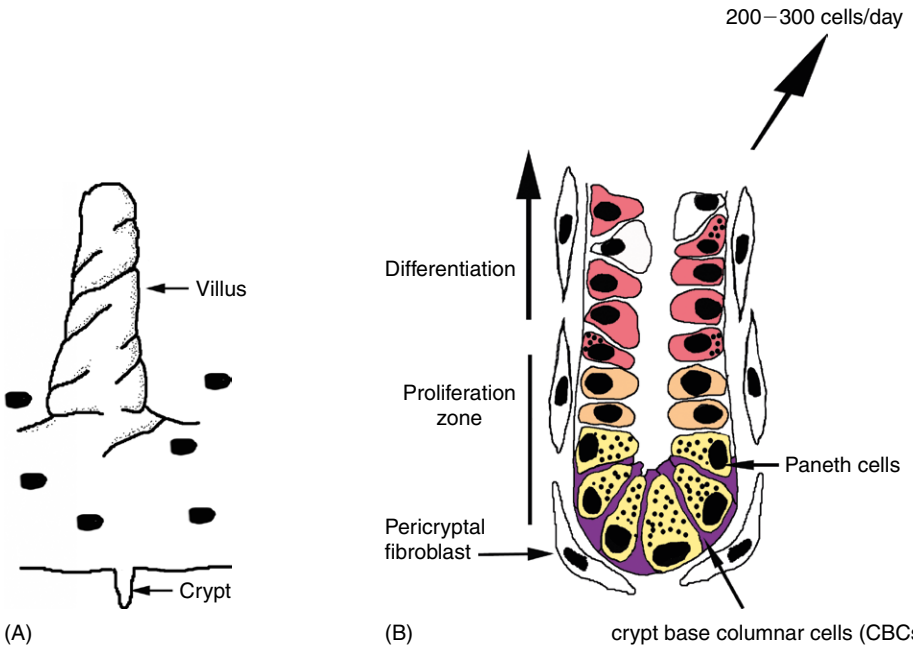


FIGURE 7-4 (A) Drawing showing the relationship between small intestinal villi and crypts. (B) Schematic representation of a small intestinal crypt. Lineage-tracing experiments in both the small intestine and the colon have revealed that the stem cells are the $Lgr5^+$ crypt base columnar cells (CBCs). In the small intestine, the CBCs are interspersed among the differentiated Paneth cells. As colonic crypts do not contain Paneth cells, the stem cells in the colon have been localized to the base of the crypt. Other differentiated cells migrate out toward the lumen of the gut. Factors secreted by the pericryptal fibroblasts could contribute to stem cell maintenance and proliferation; therefore, these cells are likely candidates for cellular components of the stem cell niche within the gut.

signal. Nuclear β-catenin is found only in the cells at the base of the crypts within the adult mouse small intestine, suggesting activation of the Wnt pathway in these cells (Figure 7-4B). Mesenchymal cells underlying the crypt epithelium could be a source of a secreted Wnt ligand that could act as a paracrine signal to direct the proliferation of stem cells, progenitor cells, or both, in the intestinal epithelium. Alternatively, Paneth cells or the stem cells themselves could provide a local source of self-renewal and survival signals.

Neural Stem Cells

Neurogenesis persists in particular regions of the adult brain, occurring in both the subventricular zone (SVZ) of the lateral ventricle and the hippocampus. Neural stem cells that have the capacity to self-renew and produce precursors that will differentiate into both neurons and glia can be cultured from the SVZ and hippocampus. When cultured *in vitro*, cells from these tissues can generate free-floating, spherical clusters called neurospheres which contain mixed populations of stem cells and precursor cells. It is likely that cells within each neurosphere provide proliferative and/or self-renewal signals that maintain proliferation of the stem cells in the inner region. Differentiating cells on the outer edges could provide maintenance signals, or the closely packed precursor cells in the center could co-stimulate each other in a form of community effect. However, it is not clear the degree to which the growth and interaction of these cells in culture resembles their interactions *in vivo* within the niche.

Astrocytes from both the SVZ and the hippocampus can provide neurogenic signals to progenitor cells, suggesting that astrocytes may be a critical component of the neural stem cell niche. However, astrocytes from the adult spinal cord have no effect on the growth of neural stem cells in culture, indicating that astrocytes from different regions of the central nervous system exhibit different capabilities for regulating the fate choice of adult stem cells. Furthermore, recent studies have shown that, similar to both spermatogonial stem cells and HSCs, neural stem cells within the SVZ reside within a vascular niche and that circulating small molecules from the blood enter the SVZ. Growth factors such as FGF-2 and EGF can support the growth of neurospheres in culture, and endothelial cells are a source of diffusible signals, such as FGF2 and VEGF (vascular endothelial growth factor) that affect neural precursors. Therefore, the vasculature is emerging as a key component of stem cell niches in mammalian tissues.

SUMMARY

Stem cell niches have been proposed to play a critical role in the maintenance of stem cells in the male germline, the hematopoietic system, the epidermis, the intestinal

epithelium, and the adult nervous system. Characterization of these stem cell niches depends on the ability to identify stem cells *in vivo* in their normal environment. Through comparison of different stem cell systems, some themes emerge that indicate possible general characteristics of the relationship between stem cells and their supporting niche.

First, secreted factors elaborated by or induced by cells composing the stem cell niche can function to direct stem cell fate decisions. However, the precise signaling pathway or pathways may be different for each stem cell type and within each stem cell niche. Studies in *Drosophila* indicate that support cells adjacent to stem cells secrete factors required for maintaining stem cell identity, and for specifying stem cell self-renewal. Both JAK-STAT signaling and TGF-β signaling have been implicated in the regulation of stem cell behavior by surrounding support cells in *Drosophila*. In mammals, the Wnt signal transduction pathway has been demonstrated to play a role in specifying stem cell self-renewal in HSCs, although the Wnt signal may be secreted from the stem cells themselves, and may act in an autocrine loop to control stem cell proliferation. Wnt signaling may also be involved in directing the proliferation of stem cells, transit-amplifying cells, or both, in the intestinal epithelium. However, the same signaling pathway may be exploited for distinct purposes in different stem cell systems. In the mammalian epidermis, Wnt signaling is likely involved in specifying the fate of hair follicle precursors, rather than in specifying self-renewal of the multipotent stem cells in the bulge.

Second, cell adhesion is also emerging as an important characteristic of the interactions of stem cells with the niche. Adhesion between stem cells and niche cells is required for stem cell maintenance in the *Drosophila* male and female germline, ensuring that GSCs are held close to self-renewal signals emanating from the niche. Attachment to niche cells, or to a basal lamina, may also be important for stem cell maintenance within adult mammalian tissues—hence the high levels of the β1 integrin characteristic of stem cells in the interfollicular epidermis and in the multipotent stem cells within the bulge region of the outer root sheath. Interestingly, targeted disruption of β1 integrin in cells within the bulge region of the outer root sheath severely impaired the proliferation of precursor cells that contributed to the interfollicular epidermis, hair follicle, and sebaceous glands. Thus, similar to the role of adherens junctions in maintaining *Drosophila* GSCs in the niche, β1 integrin-mediated adhesion may be required to hold multipotent epidermal stem cells within the niche and close to self-renewal signals. In the mammalian testis, α6 integrin has been identified as a cell surface marker for the enrichment of SSCs, although a specific role for α6 integrin in SSC maintenance has not yet been directly demonstrated. Similarly, α6 integrin is expressed by basal keratinocytes in the epidermis; however, there is no strong correlation between α6 expression and proliferative potential. Therefore, although cell adhesion is frequently a conserved feature of stem cell maintenance in supportive niches, the specific types of junctions and cell adhesion molecules that play roles may differ among different stem cell niche systems.

Third, the precise cellular organization of stem cells with respect to surrounding support cells may play an important role in the regulation of appropriate stem cell numbers. In the *Drosophila* ovary and testis, where the stem cells normally divide with invariant asymmetry, the mitotic spindle is oriented to place the daughter cell that will retain stem cell identity within the stem cell niche; the daughter cell destined to differentiate is placed outside of the niche and away from self-renewal signals. Either attachment to niche cells or the extracellular matrix via junctional complexes, or localized signals within the niche may provide polarity cues toward which stem cells can orient during division. This stereotyped division plane can in turn specify an asymmetric outcome to stem cell divisions. As stem cells are definitively identified *in vivo*, in the context of their normal support cell microenvironment, it will be interesting to determine if stem cell divisions are likewise oriented in the seminiferous tubules, bone marrow, and within neurogenic regions of the adult brain.

Understanding the roles and mechanism of action of niches in controlling stem cell behavior is critical for growing and expanding stem cells *in vitro*, while maintaining stem cell characteristics and the full array of differentiation potential, an essential first step in the use of stem cells in regenerative medicine. Recent evidence indicating that the stem cell niche changes with age and may become less capable of supporting stem cell maintenance, however, suggests that co-transplantation of new niche cells or rejuvenation of niche maintenance programs may be a necessary component of stem cell therapies used to treat older individuals. Last, understanding the interactions between stem cells and the niche may also be important for understanding tumor initiation, growth and metastasis. Emerging evidence points toward the possibility that cancer cells either find, or induce, local stromal niches that can provide signals that enable cancer cell maintenance and/or growth. Targeting the signals and pathways that promote an altered or expanded niche that supports the growth of tumor cells could provide another strategy to be used as a potent anti-cancer therapeutic. In summary, future studies that reveal the key components of stem cell niches within human tissues, in combination with bioengineering approaches to recreate niches *in vitro*, will quickly accelerate the development of stem cell-based therapies and regenerative medicine.

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KEY WORDS

Adult stem cell Relatively undifferentiated precursor cells that maintain the ability throughout adult life to proliferate, producing some progeny cells that maintain stem cell identity, renewing the stem cell population, and other progeny cells that initiate differentiation along one or more defined lineages.

APC The human *adenomatous polyposis coli* gene, a negative regulator of Wnt signaling. Also, a structural protein associated with the cytoplasmic face of adherens junctions. Mutations in the *APC* gene are linked to the development of colorectal cancer.

CySC Cyst stem cell—stem cell for the somatic cyst cells in the *Drosophila* testis.

GSC Germline stem cell.

HSC Hematopoietic stem cell.

Niche The local microenvironment that supports stem cell maintenance and self-renewal.

SSC Spermatogonia stem cell

Wnt A secreted growth factor that binds to members of the Frizzled (Fz) family of cell surface receptors.

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Chapter 8

Mechanisms of Stem Cell Self-renewal

Hitoshi Niwa

If a question can be put at all, then it can also be answered.

Ludwig Wittgenstein

INTRODUCTION

Self-renewal of embryonic stem (ES) cells is achieved by symmetrical cell division while maintaining pluripotency. This process can be modulated by extrinsic factors, such as the cytokine leukemia inhibitory factor (LIF) for mouse ES cells. External signals control gene expression by regulating transcription factors. Oct3/4 (encoded by *Pou5f1*) is pivotal in determining self-renewal or differentiation. However, the process by which self-renewal is achieved is still unclear, since cell cycle regulation, apoptosis, and telomerase activity have not been fully analyzed during ES self-renewal. More information is needed to reach a better understanding of self-renewal in ES cells.

Stem cells have qualities that seem almost mystical. Thus, we believe that, even if all possible scientific questions about these cells are answered, the primary characteristics of stem cells cannot be determined. However, as scientists, we can only ask proper questions and seek answers for them. We believe that, if questions can be posed, they can be posed clearly.

SELF-RENEWAL OF PLURIPOTENT STEM CELLS

Stem cells have the capacity for self-renewal and differentiation. Self-renewal can be defined as making a complete phenocopy through mitosis, which means that at least one daughter cell possesses the same capacity of self-renewal and differentiation. Symmetric cell division generates two stem cells with the capacity for self-renewal and differentiation, whereas asymmetric cell division results in one stem cell, and either one differentiated progeny cell or a stem cell with a restricted capacity for differentiation. Self-renewal by symmetric cell division is often observed in transient stem cells, which appear during early embryonic development, to increase body size. In contrast, self-renewal by asymmetric cell division is often observed in permanent stem cells, which appear in embryos during later stages of developmental and in adults, to maintain the homeostasis of the established body plan.

ES cells are pluripotent stem cells derived from pre- or peri-implantation embryos (Figure 8-1). The first ES cell lines were established in 1981 from the inner cell mass (ICM) of mouse blastocyst-stage embryos. A pluripotent stem cell population appears only transiently during early embryogenesis, so competency for deviation of ES cells is present during a very narrow range of developmental

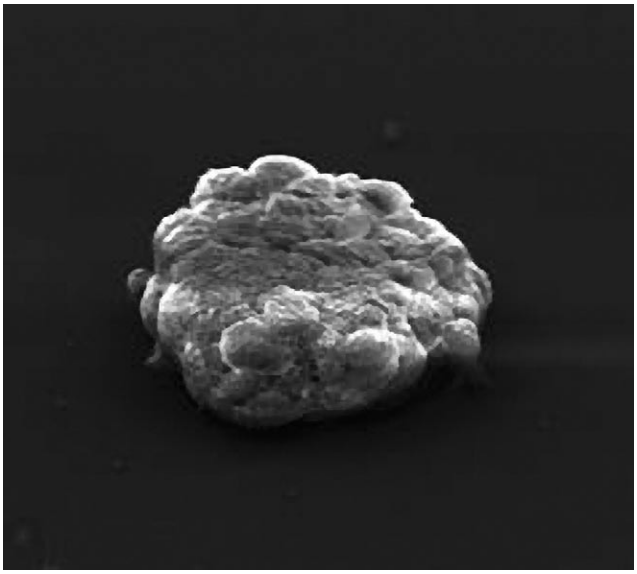


FIGURE 8-1 Scanning electromicroscope view of mouse ES cells. Mouse ES cells form compact colonies in which cells are in tight contact.

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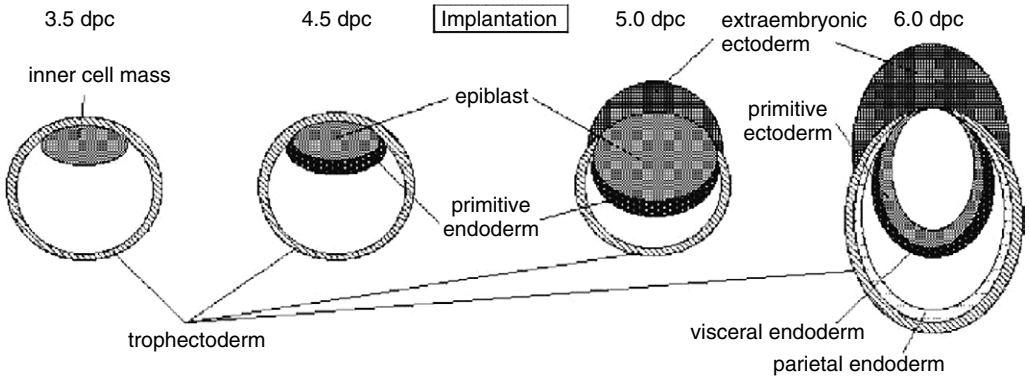


FIGURE 8-2 Peri-implantation development of mouse embryos. A pluripotent cell population is in the inner cell mass (ICM), epiblast, and primitive ectoderm. ES cells are most similar to the epiblast in their characteristics, although they are normally derived from the ICM.

stages. During mouse development, a pluripotent stem cell population is first seen as ICM by segregation of the trophectoderm during the formation of blastocysts at 3.5 days post coitum (dpc). At 4.5 dpc, a primitive endoderm layer can be seen at the surface of the ICM, and the remaining pluripotent cell population, covered by primitive endoderm, is designated as epiblast. After implantation, epiblast cells start to proliferate rapidly, and increase in size. At 6.0 dpc, apoptotic cell death eliminates the central part of the epiblast, resulting in the formation of an epithelialized monolayer of pluripotent stem cells, which is designated primitive ectoderm (Figure 8-2). Epiblast and primitive ectoderm are the sources of the other type of pluripotent stem cells, epiblast stem cells (EpiSCs) (Brons *et al.*, 2007; Tesar *et al.*, 2008). Primitive ectoderm undergoes differentiation to embryonic germ layers through gastrulation, during which process the cells lose pluripotency. After 7.0 dpc, only primordial germ cells retain latent pluripotency, which can be shown by the establishment of embryonic germ (EG) cells *in vitro* (Matsui *et al.*, 2007).

ES cells are not equivalent to the pluripotent stem cells in the ICM, although they are directly derived from the ICM. The ICM pluripotent stem cells divide slowly. In the delayed blastocyst generated by ovariectomy after fertilization, the doubling time of ICM-epiblast cells is 96 hours or longer. Mouse ES cells, however, grow more rapidly, with a doubling time of 12–14 hours. This rapid rate of pluripotent stem cell growth is observed only in the epiblast after implantation, and it may be triggered by signals from the primitive endoderm and extraembryonic ectoderm. The doubling time of these cells at 5.0 dpc is 11–12 hours, almost the same as that of ES cells, reaching 4–5 hours at 6.0 dpc. Because the epithelial characteristics and altered pluripotency are evident in primitive ectoderm, but not in ES cells, ES cells are most similar to the pluripotent stem cells in the epiblast at 5.0 dpc. Expression patterns of stage-specific marker genes, such as Rex1 (encoded by *Zfp42*), also suggest that the pattern in ES cells is most similar to the epiblast between 4.75–5.0 dpc. In addition, these cells are a heterogeneous population, containing both earlier (Rex1+) and later (Rex1−) stages of pluripotent stem cells (Toyooka *et al.*, 2008).

In the next sections, I will discuss the molecular mechanism that maintains self-renewal of mouse ES cells, and, in the final section, I will compare this mechanism to that in human ES cells.

MOLECULAR MECHANISM FOR RETENTION OF EMBRYONIC STEM CELL SELF-RENEWAL

The ability to continuously self-renew *in vitro* is one of the characteristic phenotypes of ES cells. As in other cellular phenotypes, this phenotype of ES cells is regulated by a mechanism in which extracellular signals control gene transcription in the nucleus (Figure 8-3). At the molecular level, self-renewal can be defined as a combination of maintaining pluripotency while stimulating cellular proliferation.

Extracellular Signals for Embryonic Stem Cell Self-renewal

One of the first mouse ES cell lines was established by the culture of ICM on feeder layers in the presence of medium conditioned by embryonal carcinoma (EC) cells, and another was derived from delayed blastocysts cultured on feeder cells. The role of feeder cells as sources of soluble growth factors was suggested by the efficient replacement of such cells with a medium conditioned by buffalo rat liver cells. This phenomenon was mediated by the cytokine LIF (Austin Smith *et al.*), which had been originally identified as a cytokine (Smith *et al.*) that induced differentiation and prevented self-renewal of the M1 leukemia cell line M1. In contrast, LIF had the opposite effects on ES cells, in that it inhibited differentiation while promoting the capacity

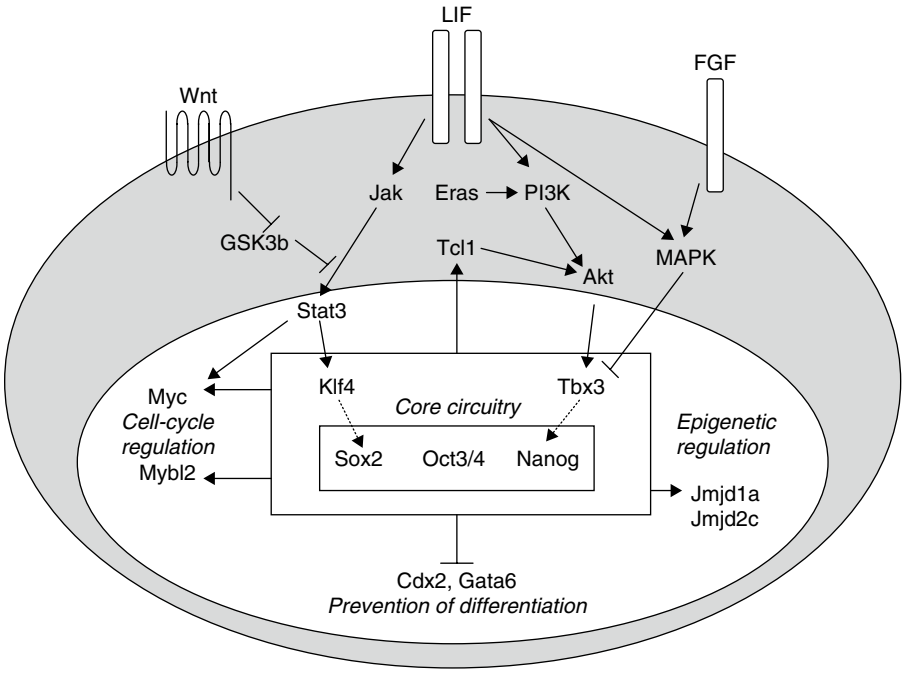


FIGURE 8-3 Molecular mechanisms governing ES cell self-renewal. The mechanism can be divided into three categories: extrinsic factors; transcriptional regulators; and effectors.

for self-renewal. Using recombinant LIF, researchers can maintain pluripotent ES cells on gelatinized dishes during long-term culture *in vitro*. Moreover, under such conditions, new ES cell lines can be established from blastocysts of the 129 genetic background, indicating that LIF alone is sufficient to maintain ES cell self-renewal.

What is the role of LIF in maintaining ES cell self-renewal? LIF belongs to the *IL-6* cytokine family, the members of which utilize the transmembrane glycoprotein Gp130 as a common component for signal transduction by their receptors. The high-affinity LIF receptor consists of a heterodimer of Gp130 and LIF receptor-β (LIFRβ). LIFRβ possesses its own cytoplasmic domain homologous to that of Gp130, but our experiment using chimeric molecules consisting of the extracellular domain of granulocyte colony-stimulating factor receptor, and the intracellular domain of either Gp130 or LIFRβ, revealed that only Gp130 is responsible for signal integration to maintain ES cell self-renewal. One of the major pathways of signal transduction that uses Gp130 is the Janus kinase (Jak)-signal transducer and activator of the transcription (Stat) pathway, which is also required to maintain ES cell self-renewal. In addition, the phosphatidylinositol-3-OH kinase (PI3K)-Akt pathway, which is also under the control of LIF, was also shown to be required, in that the artificial activation of Akt in the absence of LIF supported ES cell self-renewal.

Although LIF has a profound effect on mouse ES cells, its physiological activity during development appears to be restricted. Elimination of the function of LIF, Gp130, LIFRβ, or Stat3 by gene targeting did not interfere with the self-renewal of pluripotent stem cells during early embryogenesis. The role of Gp130 in the pluripotent cell phenotype was only evident when the delayed blastocysts were carefully analyzed. The ICM of delayed blastocysts normally maintains pluripotency, but *Gp130−/−* blastocysts could not maintain pluripotency during the delayed period. Since the maintenance of blastocysts in the uterus without implantation is a characteristic feature in rodents, the responsiveness of ES cells to Gp130 signaling likely has its origin in this adaptive physiological function. Moreover, it may be the reason that LIF does not show obvious effects on ES cells of other species, especially primates. However, the function of the Gp130–Stat3 pathway in germ cell development is evolutionally conserved, since it can be found in invertebrates. This finding suggests that the role of this system in rodent ES cells is a small evolutionary cooption derived from the maintenance of germ cells.

What about the functions of the other soluble factors? The combination of LIF and bone morphogenic factor (BMP)-4 has been reported to allow the propagation of mouse ES cells without feeder cells and FCS (Ying *et al.*, 2008). Moreover, we cultured mouse ES cells in the absence of feeder cells and serum by providing excess amounts of adrenocorticotropic hormone (ACTH) and LIF in the culture medium, indicating that ES cells require only a few factors to maintain self-renewal. The Wnt pathway may also be involved, suggesting that there may be functional overlap and crosstalk between different types of signals. Recently, ES cells were found to continue to self-renew if all the extrinsic signals were blocked by a combination of small molecule signal inhibitors (Ying *et al.*, 2008). The major target of this strategy is the mitogen activated protein kinase (MAPK) pathway activated by fibroblast growth factor (FGF) 4, which promotes the differentiation of ES

cells. When the MAPK pathway was completely blocked, ES cells continued to self-renew in the absence of LIF and FCS; in addition, their proliferation was promoted by the additional inhibition of glycogen synthase kinase 3 (GSK3). These findings indicate that self-renewal may be the ground or default state of ES cells.

Transcriptional Regulation of Embryonic Stem Cell Self-renewal

Stat3

Stat3, activated by LIF, acts as a transcriptional regulator in nuclei, but its activity is not commonly found in pluripotent stem cells. Many mouse and human EC cell lines propagate in an LIF-independent manner, as do primate ES cells, and overexpression of dominant-negative Stat3 in LIF-independent EC cells does not affect their self-renewal. These data clearly rule out the possibility that independence from exogenous LIF is not simply caused by the autocrine or intracellular activation of its signal transduction pathway. In mouse ES cells, Stat3 may primarily activate the expression of *Klf4* to maintain ES cell self-renewal (see later sections of this chapter). Among the other genes reported to be targets of Stat3 are the myelocytomatosis oncogene (*Myc*), embryonic ectoderm development (*Eed*), nuclear receptor subfamily 0 group B member 1 (*Nr0b1/Dax1*), reproductive homeobox 5 (*Rhox5/Pem*), preferentially expressed antigen in melanoma like 7 (*Pramel7*), and jumonji domain containing 1a (*Jmjd1a*).

Oct3/4

Oct3/4, initially reported as Oct3 or Oct4 and encoded by *Pou5f1*, is the first transcription factor shown to be essential for self-renewal. In 1990, three groups of researchers identified the same gene, which encodes a transcription factor of the POU family, by its specific expression in undifferentiated stem cells followed by down-regulation during differentiation. This gene is expressed in both totipotent and pluripotent cells, including fertilized eggs, all blastomeres of morula, the ICM of blastocysts, epiblast, and primitive ectoderm, but is restricted in latent pluripotent cells of the germ cell lineage, although it is expressed in differentiating cells, especially of the neural cell lineage, at 8.5 dpc. The function of *Oct3/4* in pluripotent stem cells was initially analyzed by a conventional gene-targeting strategy. Although heterozygous *Oct3/4*-deficient animals were found to develop normally, homozygous embryos exhibited developmental defects at the peri-implantation stage (Nichols *et al.*). Our subsequent functional analyses of *Oct3/4* in ES cells revealed their unique characteristics. The loss-of-function of phenotype *Oct3/4*, as evaluated by a combination of gene-targeting and tetracycline-inducible transgene expression, revealed that its function is essential for the continuous propagation of stem cell populations. Moreover, loss of *Oct-3/4* function strictly determines the differentiation of ES cells toward trophectoderm, which is observed under normal culture conditions, as found in the ICM of *Oct3/4*-null embryos. In contrast, overexpression of *Oct3/4* induces differentiation toward primitive endoderm lineage. Careful estimation of the threshold level required to induce differentiation, using tetracycline-regulatable transgenesis, revealed that a 50% increase over the normal expression level was sufficient. Overexpression of *Oct3/4* has been reported to repress its transcriptional activation using a squelching mechanism. Moreover, high-level expression of *Oct3/4* is detected in primitive endoderm imaged on the surface of the ICM at 4.5 dpc, suggesting that the phenomenon observed in ES cells may have some physiological significance. Therefore, *Oct3/4* can be regarded as a three-way switch to determine three different cell fates—pluripotent stem cells, primitive endoderm, and trophectoderm—in a dose-dependent manner. However, the artificial maintenance of *Oct3/4* expression is not sufficient to continue self-renewal without LIF.

Oct3/4 also maintains self-renewal of ES cells, preventing their differentiation toward trophectoderm, by competition with caudal-related homeobox 2 (Cdx2), a key regulator of trophectoderm differentiation. Expression of Cdx2 is immediately up-regulated after down-regulation of Oct3/4, and its overexpression in ES cells triggers differentiation to trophectoderm. Moreover, Oct3/4 represses the transcription of Cdx2 and blocks its function. Extensive bioinformatics analysis of cDNA microarrays and genome-wide chromatin immuno-precipitation (ChIP-chip) analyses identified 420 tentative target genes of Oct3/4 (Sharov *et al.*, 2008), including all of the pluripotency-associated transcription factors such as *Sox2*, *Nanog*, *Klf4*, and *Tbx3*. Interestingly, the cell cycle regulator *Mybl2* (see later section) is also included in this list, which may connect the transcription factor network with the cell cycle regulation of self-renewal.

Sox2

Sox2 is a member of the Sry-related transcription factor family. Its function in ES cells was first identified in relation to Oct3/4 during analysis of the ES-specific enhancer of the fibroblast growth factor-4 (*Fgf4*) gene. Sox2 is regarded as an Oct3/4 cofactor, since, in cooperation with Oct3/4, it activates the transcription of target genes, such as *Fgf4*, *Utf1*, *Fbxo15*, *Nanog*, and *Lefty1*. Moreover, the expression of both *Sox2* and *Oct3/4* are regulated by Oct3/4 and Sox2, indicating that a positive feedback mechanism may be involved in the maintenance of ES cell self-renewal.

Although heterozygous *Sox2*-deficient embryos generated by conventional gene-targeting develop normally, homozygous embryos stop development in the peri-implantation stage, similar to findings in *Oct3/4* mutants (Avilion *et al.*). However, *Sox-2* deficient embryos exhibit

abnormalities at a slightly later time point than *Oct3/4* mutants (abnormal embryos without an epiblast can be recovered at 6.0 dpc). Ablation of *Sox2* in ES cells was found to induce differentiation toward trophectoderm, similar to findings in *Oct3/4* mutants (Masui *et al*., 2007). Interestingly, *Sox2*-null ES cells can propagate when *Oct3/4* expression is maintained by a transgene. In these ES cells, the target genes of Oct3/4-Sox2 are normally expressed, and ChIP analyses revealed that they may be activated by other Sox family members, including Sox4, Sox1, and Sox15, which are expressed in ES cells. Therefore, the unique function of Sox2 is its activation of *Oct3/4*, and several Sox factors function as a co-factor of Oct3/4.

Nanog

Nanog (also called ENK) is a new member of the transcription factors whose functions are essential for maintaining self-renewal (Mitsui *et al.*, Chambers *et al.*, 2007). This gene, which encodes an NK2-family homeobox transcription factor, is named after Tir Na Nog, the land of the ever-young in Celtic myth, because its forced expression in mouse ES cells allows self-renewal in the absence of LIF. However, *Nanog* expression is not regulated by Stat3 directly, and *Nanog* cannot replace the function of *Oct3/4* as well as *Sox2,* so its function is still unknown. Loss of *Nanog* function in the embryo resulted in peri-implantation lethality, whereas loss of *Nanog* function in ES cells induced differentiation to parietal endoderm-like cells, along with up-regulation of Gata6. Since overexpression of Gata6 triggers a similar differentiation event, and its expression is up-regulated after withdrawal of LIF or Stat3 activity, one of the possible functions of *Nanog* may be its repression of Gata6 expression. However, ES cells were recently shown to propagate without *Nanog*, although they showed a higher incidence of spontaneous differentiation than wild-type ES cells (Chambers *et al.*, 2007).

Myc

Myc is transcriptionally-regulated by the LIF signal and overexpression of the mutant form of Myc, which does not require phosphorylation by GSK3b to promote degradation, can support the self-renewal of ES cells in the absence of LIF (Dalton *et al.*). Since *Myc* is a potent oncogene that promotes cellular proliferation, it may connect the LIF signal to promotion of self-renewal of the cell cycle.

Klf4

Klf4 is a member of the krüppel-like zinc-finger transcription factor family. We recently found that *Klf4* expression is under the control of the LIF signal via Stat3, and that overexpression of *Klf4* is sufficient to support LIF-independent self-renewal of ES cells. Functionally, Klf4 may be similar to other Klf family members expressed in ES cells, since knockdown of three Klf factors (*Klf2*, *Klf4*, and *Klf5*) prevented self-renewal of ES cells. However, Klf5 probably has a unique function since *Klf5*-null ES cells showed defects in rapid proliferation (Ema *et al.*).

Tbx3

The T-box transcription factor Tbx3 was first identified as a pluripotency-associated transcription factor by the RNAi-mediated loss-of-function screening in ES cells (Lemischka *et al.*). We confirmed that overexpression of *Tbx3* can support LIF-independent self-renewal of ES cells. *Tbx3* expression is regulated by the PI3K-Akt signal and its nuclear localization is negatively regulated by the MAPK pathway. Therefore, Tbx3 may be a target of the MAPK pathway, mediating the negative effect of the latter on self-renewal of ES cells.

Others

RNAi screening showed that *Estrogen-related receptor* β (*Esrrb*) is involved in the maintenance of pluripotency (Ivanova *et al.*, 2006), and conventional gene-targeting showed that the Spalt-related C2H2 zinc-finger transcription factor *Sall4* is involved in the rapid proliferation of ES cells (Sakaki-Yumoto *et al.*). Moreover, the C2H2 zinc-finger protein *Zfp281* and the BTB-domain containing protein *Nac1* (*Btbd14a*) are required for self-renewal (Wang *et al.*, 2006). The neuronal repressor *Rest*, which is highly-expressed in ES cells, is involved in the maintenance of pluripotency (Singh *et al.*, 2008), and the zinc-finger proteins *Zic3* and *Zfx*, the forkhead transcription factor *Foxd3*, and the HMG-box transcription factor *Tcf3* are suspected of being involved in the maintenance of self-renewal of ES cells.

Transcription Factors Involving Inner Cell Mass Outgrowth

A defect of ICM outgrowth found in *Oct3/4* or *Sox2* mutant embryos may be regarded as a landmark of gene function in pluripotent cell populations. However, such a phenotype may not always reflect the abnormality of pluripotent stem cells themselves. For example, a mutation of the signal transduction adapter protein *Disabled-2* (*Dab2*) resulted in a defect of ICM outgrowth in which only primitive endoderm cells were maintained after one week. However, *Dab2* expression was detected in primitive endoderm, but not in the ICM, and the homozygous embryos that died at 5.5 dpc exhibited abnormal migration of primitive endoderm cells. The growth defect of pluripotent cell clumps in *Dab2*-null embryos may be due to the functional deficiency of primitive endoderm cells essential for maintaining pluripotent cells as epiblasts. Similar dissociation between abnormal

phenotype in ICM outgrowth and function in pluripotent stem cells was more clearly evident in *Fgf4*. Homozygous *Fgf4*-null embryos showed a defect in ICM outgrowth, but ES cells lacking *Fgf4* function were established by serial gene-targeting. Therefore, both a cell-autonomous and a non-cell-autonomous defect can result in defective ICM outgrowth; *Foxd3* may represent the latter because its function in the differentiation of primitive endoderm has been identified in ES cells.

Effector Molecules to Retain Embryonic Stem Cell Self-renewal

Prevention of Differentiation

To maintain ES cell self-renewal, entering the differentiation process should be strictly prevented. During differentiation, expression of various genes is up-regulated in a lineage-specific manner. The GATA family transcription factors *Gata4* and *Gata6* are specifically up-regulated during differentiation to primitive endoderm induced by overexpression of *Oct3/4* or withdrawal of LIF. Their function in primitive endoderm differentiation *in vivo* has been confirmed by gene targeting. Loss of *Gata4* resulted in a functional deficiency in visceral endoderm, whereas loss of *Gata6* affected differentiation of primitive endoderm. Interestingly, ectopic expression of either *Gata4* or *Gata6* in ES cells activated the expression of endogenous Gata4 and Gata6, and induced differentiation toward parietal endoderm (Fujikura *et al.*), strongly suggesting that one of the functions of *Oct3/4* in maintaining ES cell self-renewal is the prevention of differentiation by repressing genes that induce differentiation. In the case of GATA factors, repression may occur indirectly by activation of a repressor. In contrast, genes involving trophectoderm differentiation, such as the homeobox transcription factor *Cdx2*, may be directly inhibited by *Oct3/4* because their expression is rapidly up-regulated as *Oct3/4* is repressed. We observed that ectopic expression of Cdx2 induced differentiation toward trophectoderm, indicating its significance as a gatekeeper of *Oct3/4*.

Maintenance of Stem Cell Proliferation

Proliferation of ES cells is achieved through their self-renewal. As in other cell types, proliferation should be regulated by controlling the cell cycle; however, cell cycle regulation in ES cells is unusual. For example, it may lack a major break of the cell cycle, since the retinoblastoma (Rb) gene is kept in an inactivated state by hyperphosphorylation, and transition from the G1 phase to the S phase is unlimited. In ES cells, the avian myeloblastosis viral oncogene homolog-2 (*Mybl2/ B-Myb*) was shown to be essential for normal cell cycle progression and chromosomal stability (Tarasov *et al.*, 2008). Knockdown of *Mybl2* resulted in delayed transit through G2/M, severe mitotic spindle and centrosome defects, and polyploidy. Interestingly, Mybl2 was found to directly modulate *Oct3/4* promoter activity. Conversely, *Mybl2* is a putative Oct3/4 target gene. Therefore, the reciprocal activation of *Oct3/4* and *Mybl2* may be important for the combination of pluripotency-associated transcription and cell cycle regulation.

A novel member of the Ras gene family, *Eras*, which encodes a constitutively active protein and stimulates PI3K, was recently identified by its specific expression in ES cells (Takahashi *et al.*). Loss of Eras activity in ES cells resulted in slow proliferation and poor tumorigenicity after transplantation, suggesting that its function, while not essential, is important for the rapid proliferation of mouse ES cells. A similar phenotype was observed following repression of T-cell leukemia-1 (*Tcl1*), which acts as a stimulator of Akt activity, suggesting that the PI3K-Akt pathway has a general role in the stimulation of rapid proliferation.

Unlimited propagation requires the maintenance of telomeres. ES cells possess a constitutive telomerase activity, and loss of this activity results in limited growth. ES cells lacking detectable telomerase activity, generated by the deletion of telomerase RNA, showed a reduced growth ratio after more than 300 cell divisions, and almost zero after 450 divisions. Since the induction of differentiation reduces telomerase activity, maintenance of this activity should be linked to the maintenance of ES cell self-renewal.

Regulation of apoptosis is also important for continuous growth of stem cells. Since stem cells have a proliferative activity for self-renewal *in vivo*, this activity must be strictly controlled to reduce the incidence of tumorigenesis. Compared with differentiated cells, undifferentiated ES cells show a higher incidence of apoptosis in response to various stresses, such as ultraviolet irradiation, blockage of cell cycle, and oxidative stress. Such high susceptibility to apoptosis may contribute to lower mutation rates in undifferentiated cells, in that damaged cells may be eliminated. The tumor suppressor gene *p53* contributes to hypersensitivity to UV irradiation in ES cells, but a *p53*-independent pathway may also be involved.

EPIGENETIC REGULATION OF SELF-RENEWAL

In the past few years, several epigenetic mechanisms have been identified in the maintenance of self-renewal. Mechanisms that establish and maintain silent chromatin structures (heterochromatin), such as Polycomb-related complex (PRC)-2 and RNA interference (RNAi) systems, may mediate the rapid and stable self-renewal of ES cells. In addition, nucleosome remodeling and histone deacethylation (NuRD) complex showed unique functions in ES cells. In the absence of methyl-CpG binding domain protein 3 (*Mbd3*), a component of the NuRD complex (Kaji *et al.*, 2006), ES cells failed to undergo differentiation

after withdrawal of LIF. Furthermore, Nanog and Oct3/4 associate with the unique NuRD complex to repress developmentally-regulated genes in ES cells (Liang *et al.*, 2008). However, the epigenetic mechanisms that maintain open chromatin structure (euchromatin) are also important for the maintenance of self-renewal. Jumonji-C (JmjC) domain-containing proteins act as demethylases for various types of core histones. Methylation of lysine 9 of histone H3 is a suppressive histone marker that triggers the recruitment of heterochromatin protein 1 (HP1). This modification does not occur in the presence of JmjC domain containing proteins, such as Jmjd1a and Jmjd2c. In addition, *Jmjd1a* and *Jmjd2c* are required for the proper maintenance of ES cell self-renewal of ES cells, and their expression is regulated by Oct3/4 (Loh *et al.*, 2007). Acethylation of histones H3 and H4 are involved in active transcription, and the p400/Tip60 histone acethyltransferase complex may be associated with ES self-renewal (Panning *et al.*). Recently, a novel epigenetic modifier *Ronin* (*Thap14*) was shown to be an essential and sufficient factor for maintaining ES cell self-renewal (Zwaka *et al.*). This gene encodes a Thap-domain zinc-finger DNA binding protein, and directly interacts with host cell factor 1 (HCF-1), which is associated with histone methyltransferases Set1 and Mll1, leading to the trimethylation of lysine4 on histone H3, which is a component of active chromatin.

SELF-RENEWAL OF HUMAN EMBRYONIC STEM CELLS

Human ES cells show various characteristics that differentiate these cells from mouse ES cells. They grow as a monolayer sheet, and attach to the culture surface even in the presence of feeder cells. Their growth ratio is slower than that of mouse ES cells, and they require FGF2 and Activin/Nodal signals instead of the LIF signal to continue self-renewal. Since mouse EpiSCs share these characteristics, it is likely that the developmental state of human ES cells is similar to that of the late epiblast/primitive ectoderm, rather than the early epiblast of mouse ES cells. If so, the mechanisms that maintain self-renewal of mouse ES cells should not be shared completely by human ES cells, and careful comparisons of mouse ES cells, mouse EpiSCs, and human ES cells will be required.

SUMMARY

ES cell self-renewal has been analyzed over the past two decades using mouse ES cells, and many principal molecules have been identified. The isolation of human ES cells has accelerated studies on ES cells of various organisms except rodents, revealing that ES cell lines of different origins have both common and different characteristics. Since there are some differences in the mechanisms required to maintain self-renewal of human and mouse ES cells, we will need to further characterize human ES cells and compare them to mouse ES cells, to determine their future application in regenerative medicine. However, although our understanding of the molecular mechanisms governing self-renewal is far from complete, ES cells can be regarded as among the best characterized stem cells. Therefore, results from ES cell studies can provide good models for other stem cell systems.

KEY WORDS

Pluripotency An ability to differentiate into a variety of cells that belong to all three germ layers, with at least one cell type for each. In contrast, totipotency is defined as the ability to generate a whole animal autonomously, and multipotency as the ability to yield multiple cell types that belong to particular germ layers, not all three. Unipotency is defined as the ability to generate a single differentiated cell type, the most limited ability of stem germline stem cells.

Self-renewal A style of cell division characteristic of stem cells that yields at least one daughter cell with the same differentiation ability as the parental stem cells. Symmetric self-renewal generates two stem cells whereas asymmetric self-renewal generates one stem cell and one differentiated progeny cell. At the molecular level, this event can be divided into two mechanisms—preventing differentiation and promoting cell division.

Stem cell A cell that has the ability to self-renew and differentiation. Stem cells can be found as transient populations during development and as stable populations in adult tissues. Embryonic stem cells are specified as stem cells derived from the pluripotent stem cells of early-stage embryos.

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Chapter 9

Cell Cycle Regulators in Adult Stem Cells

Tao Cheng and David T. Scadden

INTRODUCTION

Adult stem cells have defined therapeutic roles evident in clinical hematopoietic stem cell (HSC) transplantation. Therapeutic efficacy of stem cells in part depends on their proliferation; therefore, strategies to manipulate them require understanding of their cell cycle control. A significant hurdle restricting broader use of adult stem cells is their limited number and differentiation in response to proliferative stimuli, thus compromising *ex vivo* expansion efforts. Cell cycle regulators play important roles in this process. In this chapter, we do not intend to detail the biochemical pathways of general cell cycle regulation because they were largely obtained from other model systems and have been extensively reviewed elsewhere. Instead, we focus on the distinct cell cycle kinetics in stem cell populations and its molecular base, exemplified by the defining roles of the CKIs in murine HSCs. Admittedly, those studies do not give the whole picture with regard to how the cell cycle in stem cells is controlled. Nevertheless, they underscore the importance of further investigation of other cell cycle regulators in stem cell biology and offer new paradigms for therapeutic manipulations of stem cells.

CELL CYCLE KINETICS OF STEM CELLS *IN VIVO*

As largely modeled in the hematopoietic system, maintenance of mature cell production requires a cytokine-responsive progenitor cell pool with prodigious proliferative capacity and a much smaller population of stem cells intermittently giving rise to daughter cells, some of which constitute the proliferative progenitor compartment. Under activating conditions, such as after transplantation, an increase in stem cell divisions takes place, as evidenced by depletion of cycling cells using S-phase toxins (5-fluorouracil [5-FU] or hydroxyurea). However, in most systems relative quiescence or slow cycling in the stem cell pool appears to be essential to prevent premature depletion under conditions of physiological stress over the lifetime of the organism. Therefore, the highly-regulated proliferation of HSCs occurs at a very limited rate under homeostatic conditions.

HSC proliferation has been directly measured by bromodeoxyuridine (BrdU)-labeling experiments, and cell cycle lengths have been estimated at approximately 30 days in small rodents, or only about 8% of the cells cycling daily. However, recent studies call into question the validity of BrdU labeling and experiments employing retention of other dyes demonstrate a dormant population of HSC that cycle approximately every 145 days. Analyses using population kinetics in cats have estimated that stem cells replicate once every 10 weeks. In higher order primates, the frequency of cell division in the stem cell pool has been estimated to occur once per year. However, it is still not clear whether the relative quiescence reflects a complete cell cycle arrest of most cells in the stem cell compartment, termed the "clonal succession model," or a very prolonged G1 or G2 phase of cycling stem cells. Although the retrovirus-based clonal marking studies indicated a dormancy of most HSCs at a given time, which supports the clonal succession model, this view has been challenged by the competitive repopulation model and by BrdU incorporation in a defined HSC pool.

In contrast, the essential feature of the progenitor population (such as the hematopoietic progenitor cell, HPC) is that it irreversibly develops into maturing cells, and in the process undergoes multiple, rapid cell divisions. The progenitor cell pool essentially operates as a cellular amplification machine, generating many differentiated cells from the few cells entering the system. Therefore, it is directly responsible for the number of terminally differentiated cells. It is also termed the transit, amplifying cell pool. The differences between the stem and progenitor cell populations in different stages have been regarded as a phenotype distinction, marking the stage of a cell within the hematopoietic cascade. However, an alternative model recently proposed is that the specific position in a cell cycle determines whether a primitive cell functions as a stem cell or a progenitor cell. In this model, stimuli received at distinct positions in the cell cycle provoke proliferation–differentiation, and yield

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either stem or progenitor cell outcomes, thereby challenging the traditional view of "hierarchy" within the hematopoietic differentiation.

In either model, "stemness" associates with a limited rate of cell proliferation. This model may not be uniform however. Even within the hematopoietic system there is heterogeneity with some stem cells being deeply dormant (approximately 20% of murine HSC) and others more rapidly cycling. Shifting between more or less quiescence occurs under particular physiologic conditions.

In other tissues such as the central nervous system (CNS), evidence suggests that the proliferative pools of adult neural progenitors are derived from a quiescent multipotent precursor cell or neuronal stem cell (NSC). Ablation of the proliferative zone containing the lineage-committed neuronal progenitor cell (NPC) can be repopulated from a small number of quiescent NSCs. In the mouse dermal stem cell population, there are about four-fold fewer cells in S-G2/M in the stem cell population compared with the progenitor pool, although both cell populations constantly proceed through the cell cycle. In some settings, however, stem cells may actively cycle. The intestinal stem cell identified by Lgr5, for example, cycles rapidly. This cell clearly functions as a stem cell, however, whether there is another population of more quiescent stem cells in the intestine also has experimental support. There may therefore be heterogeneity in stem cell pools in multiple tissues with one pool serving as a deeply quiescent reserve and another more rapidly proliferating pool providing more daughter cells for tissue maintenance.

STEM CELL EXPANSION *EX VIVO*

The relative quiescence of stem cells may prevent their premature exhaustion, but it is problematic in the context of the *in vitro* expansion necessary for transplantation and gene therapy. Methods for inducing stem cell proliferation have long been sought as a means to expand the population of cells capable of repopulating the marrow of ablated hosts, and to render stem cells transducable with virus-based gene transfer vectors. Although great effort has been made to directly expand stem cells using different combinations of hematopoietic growth factors (cytokine cocktails), few culture systems have been applied in the clinical setting, at least in part because of the lack of proof that any of the culture conditions support expansion of long-term repopulating HSCs in humans. Gene-marking studies in large animals, including primate and human, indicate poor transduction in the stem cell compartment during long-term engraftment. These cytokine-based efforts to expand stem cells have often resulted in increased cell numbers, but at the expense of multipotentiality and homing ability. Although data suggest that under some specific conditions murine HSCs may divide *in vitro*, net expansion is achieved in a limited fashion, and is always associated with, and often dominated by, cellular differentiation. Recent studies on the potent effects of Notch ligands and Wnt proteins on HSC expansion *in vitro* are promising. However, whether such "successful" protocols can be adapted to clinically useful human HSC expansion remains to be determined.

Alternative approaches aimed at targeting the negative regulatory cytokines have also been sought to activate or expand stem cells. Factors such as TGFβ-1 and macrophage inhibitory protein-1α (MIP-1α) have been noted to play a role in dampening hematopoietic cell growth kinetics. In particular, TGFβ-1 has been shown to be able to selectively inhibit the growth of HSCs and progenitor cells. Antisense oligonucleotides, or specific neutralizing antibodies, have been shown to permit primitive hematopoietic cell entry into the cell cycle, and to enhance the efficiency of retroviral transduction into those cells. However, long-term engraftment after *in vitro* manipulation by these methods remains to be defined in an *in vivo* model. More efficient and specific methods, such as RNA interference technology, aimed to knock down the essential elements in the inhibitory circuits of stem cell expansion are at the moment the most promising development.

Although HSCs may be induced to divide *in vitro*, it remains unclear which combination of factors is specific for stem cell proliferation without differentiation. The complex microenvironments ("niches") in which the stem cells reside and the intrinsic properties of HSCs in relation to the environmental cues are largely unknown. The ultimate success of stem cell expansion *in vitro* will require greater understanding of the interplay between stem cell and microenvironment, and the signaling circuitry involved in achieving self-renewal.

MAMMALIAN CELL CYCLE REGULATION AND CYCLIN-DEPENDENT KINASE INHIBITORS

The molecular principles of cell cycle regulation have been largely defined in yeast, and with orthologous systems it is applicable to the mammalian cell cycle. A number of surveillance checkpoints monitor the cell cycle and halt its progression, mainly via the p53 pathway, when DNA damage occurs and cannot be repaired. In eukaryotic cells, factors that determine whether cells will continue proliferating or cease dividing and differentiate appear to operate mainly in the G1 phase of the cell cycle (Figure 9-1). Cell cycle progression is regulated by the sequential activation and inactivation of CDKs. In somatic cells, movement through G1 and into S phase is driven by the active form of the Cyclin D1, 2, 3/CDK4, 6 complex and the subsequent phosphorylation of retinoblastoma (Rb) protein. Once Rb is phosphorylated, the critical transcription factor, E2F-1, is partially released from an inhibited state and turns on a series of genes, including cyclin A and cyclin E, which form a complex

with CDK2 and cdc25A phosphatase. The cdc25A is able to remove the inhibitory phosphates from CDK2, and the resultant cyclin E/CDK2 complex then further phosphorylates Rb, leading to a complete release of E2F and the transcription of multiple other genes essential for entry into S-phase and DNAsynthesis. In parallel, the c-myc pathway also directly contributes to the G1–S transition by elevating the transcription of genes for cyclin E and cdc25A (Figure 9-1). CDK activity is strictly dependent on cyclin levels that are regulated by ubiquitination and subsequent proteolysis. On mitogenic stimulation, cyclin D serves as an essential sensor in the cell cycle machinery and interacts with the CDK4/6-Rb- E2F pathway.

In addition to regulation by cyclins and phosphorylation/dephosphorylation of the catalytic subunit, CDKs are largely controlled by CKIs. Two families of low molecular weight CKIs, Cip/Kip and INK4, are capable of interacting with CDKs to impair progression through the G1 phase. The Cip/ Kip family, which includes p21Cip1/Waf1, p27kip1, and p57Kip2 (p21, p27, and p57 hereafter), may interact with a broad range of cyclin–CDK complexes, whereas the INK4 family, p16INK4A, p15INK4B, p18INK4C, and p19INK4D (p16, p15, p18, and p19 hereafter), specifically inhibit CDK4 and CDK6 kinases. Both families have been shown to have essential roles in arresting cell cycle progression in a number of model systems. Studies using antisense strategies have been able to release the cells in G0 stage into the cell cycle. Knockout analysis in rodent models has provided a strong basis for further exploring those molecules in stem cell biology. Interestingly, the CKIs p27 and p18 have a profound effect on overall cellularity and organ size, resulting in a larger whole animal when either gene is knocked out. There appears to be a distinct cell cycle control operating in stem cells to maintain their stemness. This has been shown in mouse embryonic stem (ES) cells with a "defective" Rb pathway. In somatic tissues, because stem, progenitor, and more differentiated cells share many common cytokine receptors, it is likely that the distinct cell cycle profile in stem cells must be mediated by either distinct upstream intracellular mediators, or unique combinatorial relationships of common biochemical mediators, limiting the intensity of signals to enter into cell cycle. Defining the basis for the participating mechanisms in the stem cell response requires stepwise analysis of individual cell cycle regulators and, ultimately, a systems approach to define how these cell cycle regulators interact with each other and react to upstream signaling molecules in multiple pathways.

ROLES OF CYCLIN-DEPENDENT KINASE INHIBITORS IN STEM CELL REGULATION

Although significant progress has been made in our understanding of how the cell cycle is regulated in a variety of other model systems, limited knowledge has been gained about how the cell cycle is molecularly controlled in stem cells. Given the relative quiescence of stem cells *in vivo*, a reasonable starting point is the analysis of cell cycle inhibitors and whether reduction of their cell cycle blockade may be a mechanism for enabling stem cell entry into the cell cycle (Figure 9-2).

CKIs have been demonstrated to be involved in a number of stem and progenitor cell systems. *Dipio*, an analog of p21/p27 in *Drosophila*, has been reported to control embryonic progenitor proliferation. Recent studies of knockout mice and cells that lack a specific CKI have begun to clarify their unique activity in stem cell populations. An increased stem or progenitor cell potential has been found in p21_/_ or p27_/_ mice when dermal, neural, or otic tissues were assessed. In the hematopoietic system, most CKI family members have been found to be expressed in CD34_ cells, although the expression patterns differ. Functional assessments of several CKIs (including p21, p27, p16 and p18) in stem cells (especially HSCs) have been carried out using the _/_ mice.

Roles of p21 in Stem Cell Regulation

In the absence of p21, there has been conflicting evidence of effect depending on the mouse strain studied. In a mixed genetic background, stem cell cycling seemed to

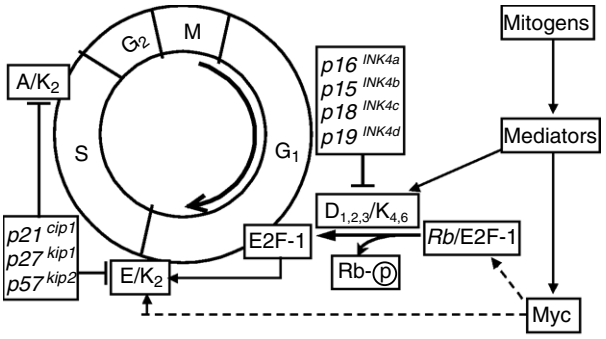


FIGURE 9-1 Cell cycle regulators in G_1 and S phases (description detailed in the text).

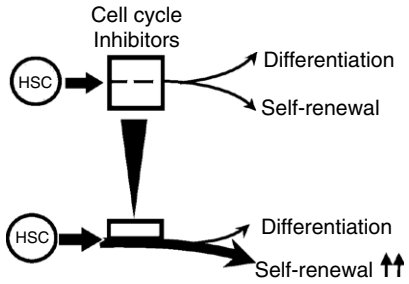


FIGURE 9-2 Model of targeting cell cycle inhibitors for enhancement of stem cell self-renewal. *Top*: Under the homeostatic condition. *Bottom*: Potential novel approach for stem cell expansion by targeting the cell cycle inhibitors.

be increased. Exposing the animals to cell cycle-specific myelotoxic injury resulted in premature death because of hematopoietic cell depletion. Furthermore, self-renewal of primitive cells was impaired in serially transplanted bone marrow from p21_/_ mice (129/SV background) leading to hematopoietic failure. These data suggested that p21 governs cell cycle entry of stem cells, and, in its absence, increased cell cycling leads to stem cell exhaustion. However, on a pure C57B1/6 background stem cell cycling and number were unaffected. Therefore, there may be critical other genetic determinants of p21 function in HSC.

Under conditions of stress, it appears that restricted cell cycling is crucial to prevent premature stem cell depletion and hematopoietic death. This paradigm has been also shown in the mice deficient in Gfi-1, Pten, or Foxo proteins. However, the specific effect of p21 on the self-renewal outcome of divided HSCs seems also to depend on other important factors, such as HoxB4 and certain genetic modifiers. Nevertheless, the positive effect of p21 absence on mouse HSCs has been extended to human cells and to a nondevelopmental context. Using postnatal CD34_ CD38_ human cells, it was shown that interrupting p21 expression with lentivectors *ex vivo* resulted in expanded stem cell numbers, which was validated by increased function in the transplantation assay using irradiated NOD-SCID mice recipients. Such a study further supports an alternative paradigm for increasing HSC numbers by releasing the brake on cell cycle entry, rather than focusing on a combination of pro-proliferative cytokines. Importantly, these data further supported the notion that postnatal human stem cell proliferation can be uncoupled from differentiation in *ex vivo* settings.

Interestingly, the role of p21 has been paradoxically noted to positively affect proliferation capability following cytokine stimulation in progenitor cell pools. This may be due to the requirement for p21 to promote the association of CDK4 with D-type cyclins. LaBaer and colleagues demonstrated that low concentrations of p21 promote assembly of active kinase complexes and thereby entry into cycle, whereas higher concentrations are inhibitory. The stoichiometry of p21 and cyclin–CDK complexes appears to be crucial in determining the relative effect on movement of the cell through late G1 into S phase. This was further confirmed in a study showing that p21 and p27 are essential activators of cyclin D-dependent kinases in murine fibroblasts. Mantel *et al*. noted that bone marrow progenitor cells from mice proliferated poorly and formed few colonies with thymidine treatment, except when transduced with a p21-encoding retroviral vector. Similarly, we noted a transient rise in p21 immediately following release of cell cycle arrest in 32D cells. Therefore, as observed in other systems, p21 has a dual function in the hematopoietic system, depending on the differentiation stage and CDK complex type and status. In addition, complex roles of p21 in apoptosis or differentiation may participate in stem cell regulation, although these functions are yet to be thoroughly investigated.

Why p21 expression is elevated in HSCs is unclear, but two upstream regulators have been assessed. WTI is known to induce p21 transcription, and overexpression of WTI results in altered stem cell cycling and differentiation in primary hematopoietic cells. However, null-mutant mice for WTI do not appear to have a stem cell defect. It is well known that p21 is also transcriptionally regulated by p53 and serves as a downstream mediator of cell cycle arrest induced by the p53 pathway. Interestingly, in the absence of p53, HSC function has been reported to be significantly enhanced under stress conditions in a manner opposite to that in the absence of p21. Because p53 mediates apoptosis in many cell types, the enhanced function of HSCs in the absence of p53 suggests that in some settings (e.g., stress) increased survival may dominate over accelerated proliferation of HSCs.

Roles of p27 in Stem Cell Regulation

Of particular interest to tissue regeneration is p27, because of its direct involvement in cell cycle-mediated hyperplasia. Direct flow cytometric analysis shows p27 expression in primitive cells and in more mature progenitors, supporting the hypothesis that p27 has a role in hematopoiesis. This role is also supported indirectly by improved retroviral transduction following knockdown of p27 with an antisense oligonucleotide. The p27 appears to accumulate at points in which signals for mitosis affect cell cycle regulators, and has been shown to serve as an important regulator at a restriction point for mitogenic signals. Because progenitor cells have a robust response to growth factors, p27 likely plays a specific role in progenitor cell pools. Its role may be quite distinct from that of p21, which is shown to be a molecular switch for stem cell cycle control. Disruption of the p27 gene has resulted in a mouse with hyperplasia of a number of organs (including hematopoietic tissues) and spontaneously generated tumors of specific type.

Using p27_/_ mice, researchers have reported that p27 does not primarily affect stem cell number, cell cycling, or self-renewal, but markedly alters progenitor proliferation and pool size. When competitively transplanted, p27-deficient stem cells generated progenitors that eventually dominated blood cell production. Thus, modulating p27 expression in a small number of stem cells may translate into effects on most mature cells, thereby providing a strategy for potentiating the impact of transduced cells in stem cell gene therapy. Such a dramatic effect of p27 absence on hematopoietic reconstitution was also observed in liver regeneration, and a specific role of p27 in the committed progenitor cells not at the stem cell level was also reported in the mouse CNS. Therefore, distinct roles for p27 and p21 have been defined in hematopoiesis, and indirect evidence suggests that these distinctions may be preserved across stem and progenitor pools from multiple different adult tissues.

Other Cyclin-dependent Kinase Inhibitors and the Retinoblastoma Pathway in Stem Cell Regulation

One of the best studied pathways in cell cycle regulation is that of Rb, which directly interacts with Cyclin D and the INK4 proteins in early G1 phase and serves as a critical and initial interface between mitogenic stimuli and cell fate commitment following division. A role for Rb in stem cell regulation is indirectly supported by the finding that ES cells do not have intact G1 machinery, but that acquisition of Rb pathway products induces the transition from symmetric to asymmetric cell division, which is a critical feature of mature stem cell function. Mice deficient in Rb are not viable and show defects in multiple tissue types, including the hematopoietic lineage. After conditional deletion of Rb in mice, malfunctioning of HSC was recently documented, that appeared to be due to microenvironmental and/or intrinsic defects.

The INK4 proteins closely associated with Rb have been studied in the context of stem cell biology. These studies include several reports indicating that Bmi-1, an upstream inhibitor of p16 and p19ARF (ARF), is critical for HSC selfrenewal. In the absence of Bmi-1, self-renewal of HSCs and neural stem cells is diminished, an effect dependent on the expression of p16. In mice engineered to be devoid of p16, a complex HSC phenotype has been observed, with a decreased number of stem cells in younger mice, but enhanced self-renewal in serially transplanted animals. A similar anti-aging effect of p16 deletion was reported for neural and pancreatic islet cells.

The INK4 family member, p18, is expressed in multiple tissue types including hematopoietic cells, the loss of which in mice results in organomegaly with higher cellularity and an increase in the incidence of tumors with advanced age or in the presence of carcinogens. Furthermore, it has been suggested that p18 is involved in the symmetric division of precursor cells in mouse developing brain. Recently, it has been reported that the absence of p18 increases HSCs, similar to the p21 null setting. But unlike p21 absence, an increase in stem cell self-renewal is observed in the absence of p18.

Systematic evaluation of proximate molecular regulators of cell cycling is, therefore, yielding a complex picture of how each influences primitive cell function. Different members of the CKI subfamilies appear to play distinct roles in stem or progenitor cell populations. The function of these different CKIs appears to be highly differentiation-stage specific and confers an important level of regulation in stem or progenitor cells to maintain homeostasis. Cooperative effects between members of the two CKI subfamilies are likely, with evidence of such interplay between p15 and p27 now documented. How the CKIs exert distinct effects, and the pathways converging on these regulators, are the subject of ongoing study and will potentially provide further insight for manipulation of stem and progenitor populations. Whether these pathways are shared among primitive populations of cells in all tissues is not yet clear, but preliminary data suggest that such is the case.

Relation Between Cyclin-Dependent Kinase Inhibitors and Transforming Growth Factor β-1

TGFβ-1 has been documented to have varied effects on hematopoietic cells, including enhancement of granulocyte proliferation in response to granulocyte-macrophage colony-stimulating factor, or inhibition of progenitor cell responsiveness to other growth-promoting cytokines. The detailed roles of TGFβ-1 in signaling pathways and in hematopoiesis have been extensively reviewed elsewhere. TGFβ-1 has been extensively characterized as a dominant negative regulator of hematopoietic cell proliferation, including inhibiting primitive progenitor cells. Antisense TGFβ-1 or neutralizing antibodies of TGFβ-1 have been used to induce quiescent stem cells into the cell cycle, and to augment retroviral gene transduction in conjunction with down-regulation of p27 in human CD34_ subsets. Based on the roles of CKIs in hematopoietic cells as described previously, the link between TGFβ-1 and CKIs in stem cell regulation has been recently addressed. TGFβ-1-induced cell cycle arrest has been shown to be mediated through p15, p21, or p27 in multiple cell lines or cell types, including human epithelial cell lines, fibroblast cells, and colon and ovary cancer cell lines. Recently, it was proposed that p21 and p27 are key downstream mediators for TGFβ-1 in hematopoietic cells, and a study examined whether p21 or p27 was a proximal mediator for TGFβ-1-induced cell cycle exit in primary hematopoietic cells. Using fine mapping of gene expression in individual cells, researchers documented TGFβ-1 and p21 to be up-regulated in quiescent, cytokine-resistant HSCs, and also in terminally differentiated mature blood cells as compared with immature, proliferating progenitor cell populations. Type II TGFβ-1 receptors were expressed ubiquitously in these subsets of cells without apparent modulation.

To provide further biochemical analysis of whether the coordinate regulation of TGFβ-1 and p21 or p27 represented a dependent link between them, the cytokine-responsive 32D cell line was analyzed for p21 or p27 up-regulation following cell cycle synchronization and release in the presence or absence of TGFβ-1. Despite marked antiproliferative effects of TGFβ-1, neither the transcription of p21 mRNA nor the expression of p21 or p27 was altered. To corroborate these observations in primary cells, bone marrow mononuclear cells derived from mice engineered to be deficient in p21 or p27 were assessed. Both progenitor and primitive cell function were inhibited by TGFβ-1 equivalently in knockout and wild-type littermate controls. This data indicated that TGFβ-1 exerts its

inhibition on cell cycling independent of p21 and p27 in primitive hematopoietic cells.

Other data have recently reported examining the Cip/Kip CKI family member p57 in hematopoietic progenitors. The absence of p57 was associated with a lack of responsiveness to TGFβ, failing to arrest cell cycling. Furthermore, TGFβ was noted to induce p57 expression, arguing for a direct link between TGFβ and the cell cycle regulatory function of p57. In addition, Dao *et al*. reported that blocking TGFβ-1 could down-regulate p15 expression in human CD34_ cells and that TGFβ-1 may act through the INK4 family and the Cip/Kip family in hematopoietic cells. However, extensive biochemical analysis in primary hematopoietic cell subsets is needed to further address this question.

CKIS and Notch

Notch1 has been well-defined as a mediator of decisions at multiple steps in the hematopoietic cascade, including stem cell self-renewal versus differentiation. Variable effects on cell cycling have been reported, including inhibition of proliferation and, in contrast, maintenance of proliferation but with a decreased interval in the G1 phase of the cell cycle. The latter observation has been followed up by more extensive analysis of an interaction between Notch1 and CKI regulation. It has been reported that the basis for Notch influencing G1 may be through alteration in G1–S checkpoint regulator stability, specifically affecting the proteosome degradation of p21 and p27. The links of receptormediated effectors of stem cell function and cell cycle regulators are, therefore, beginning to emerge and provide an essential component of the larger regulatory network in stem cells, as exemplified in the Notch pathway.

SUMMARY

Further therapeutic potential of stem cells is envisioned to be broadened if the biology of stem cells can be exploited to permit efficient *ex vivo* manipulation and enhance repopulation *in vivo*. Given the relative quiescence of HSCs, which has not been satisfactorily overcome by cytokine manipulation *in vitro*, direct intervention in the control of the cell cycle has been sought as an approach to disassociate cell proliferation from cell differentiation, thereby potentially bypassing a major hurdle in current stem cell expansion strategies. CKIs appear to be compelling candidates for the latter approach. Targeting specific CKIs together with TGFβ-1 may provide complementary strategies for enhancing hematopoietic stem or progenitor cell expansion and gene transduction. Controlled manipulation of specific CKIs directly, or through their upstream signaling molecules such as Notch1, may also be relevant for the expansion or possible regeneration of other non-HSC pools.

As the roles of cell cycle regulators in the molecular control of stem cells are explored, many issues remain to be addressed. Much work is needed to delineate the roles of individual members of the cell cycle machinery within the context of tissue-specific stem cell types. Furthermore, how these relate to one another and to signal transduction pathways that operate uniquely in specific stem cell populations has yet to be elucidated. Coupling extrinsic signals to cell cycle control will be essential steps before understanding how a stem cell responds to the complicated setting of its microenvironment. Piecing together the components and their interactions, by either a reductionist or systems approach, will offer targets for a more rational manipulation of the stem cell.

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KEY WORDS

CDK inhibitors (CKIs) Intracellular molecules with low molecular weight specifically inhibiting the activities of cyclin-dependent kinases during cell cycle progression. There are two subfamilies of CKIs, including Cip/Kip (p21Cip1/Waf1, p27kip1, and p57Kip2) and INK4 (p16INK4A, p15INK4B, p18INK4C, and p19INK4D).

Stem cell quiescence Mitotic quiescence or slow cycling of adult stem cells in comparison with the lineagecommitted progenitor cells. It is generally associated with G0 or prolonged G1 phase in cell cycle.

Stem cell self-renewal The process by which a stem cell replicates itself. It is not a synonym of stem cell proliferation since the proliferation may be also accompanied by cell differentiation.

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Chapter 10

Epigenetic Mechanisms of Cellular Memory During Development

Nathan Montgomery, Terry Magnuson, and Scott Bultman

INTRODUCTION

Chromatin and Transcriptional Regulation During Development

Regulation of gene expression is inherently more complicated in eukaryotes than prokaryotes, because the transcriptional machinery must recognize a chromatin template instead of naked DNA (Figure 10-1). Consequently, chromatin-modifying factors must play crucial roles in transcriptional regulation, and be involved in the ability of stem cells to proliferate and differentiate into diverse cell types that have an identical genome sequence. This supposition is supported by the fact that mutations in chromatin-modifying factors generated by gene targeting in the mouse confer mutant phenotypes ranging from early embryonic lethality to neoplasia (Li, 2002). As described below, the functional significance of chromatin-modifying factors is also clearly demonstrated by normal and abnormal epigenetic events that have been documented in somatic cell nuclear transfer (SCNT) experiments.

The Importance of Chromatin in Heredity is Exemplified by Unexpected Coat Colors and Developmental Defects in Cloned Animals

The first cloned cat, called Cc for either copy cat or carbon copy, is interesting because she has different coat-color markings than her genetic donor, despite being genetically identical. This observation indicates that DNA sequence alone is not sufficient to confer certain genetic traits. Instead, a heritable influence must exist that is not based on DNA sequence. This influence, referred to as epigenetics, is not as well-understood as the genetic code, but it is becoming increasingly clear that it involves the ability of chromatin structure to affect transcription at the level of individual genes, clusters of genes (particularly imprinted genes), or even whole chromosomes. In the case of Cc, the apparent paradox of her unexpected coat-color markings can be reconciled by an epigenetic event called X-chromosome inactivation (XCI). Female mammals have two X chromosomes, whereas males have only one. Consequently, one of the two X chromosomes is inactivated stochastically on a cell-by-cell basis in females during early embryogenesis and maintained in a clonal manner throughout the numerous cell divisions that occur during development. The primary result of this process is to achieve the same level of X-linked gene activity in females as males (i.e., dosage compensation), but the stochastic nature of XCI also results in individual females having unique distributions of cells where one or the other X chromosome is inactivated. This functional mosacism is usually not visibly evident, but it can be readily observed in Cc because her two X chromosomes carry different alleles of a pigmentation gene partly responsible for calico coat color. The allele conferring orange color was inactivated in a different subset of pigment producing cells (i.e., melanocytes) than the cat from which she was derived, so Cc had a different pattern of orange, black, and white coloration.

Not surprisingly, SCNT is a very inefficient process, compared to *in vitro* fertilization or more conventional embryological manipulations. In fact, Cc was the only clone to survive out of over 200 attempts, which is consistent with success rates of less than 1% and up to 3% for other mammals in most SCNT experiments. Many clones fail to reach the blastocyst stage and successfully implant into the uterus (Hochedlinger and Jaenisch, 2006). Clones that progress beyond this stage often die *in utero* because of placental defects, or die shortly after birth with large offspring syndrome, which includes increased birth weight, respiratory distress, and/or cardiovascular defects. Furthermore, although it is too soon to assess the long-term health of cloned cattle, sheep, goats, pigs, and

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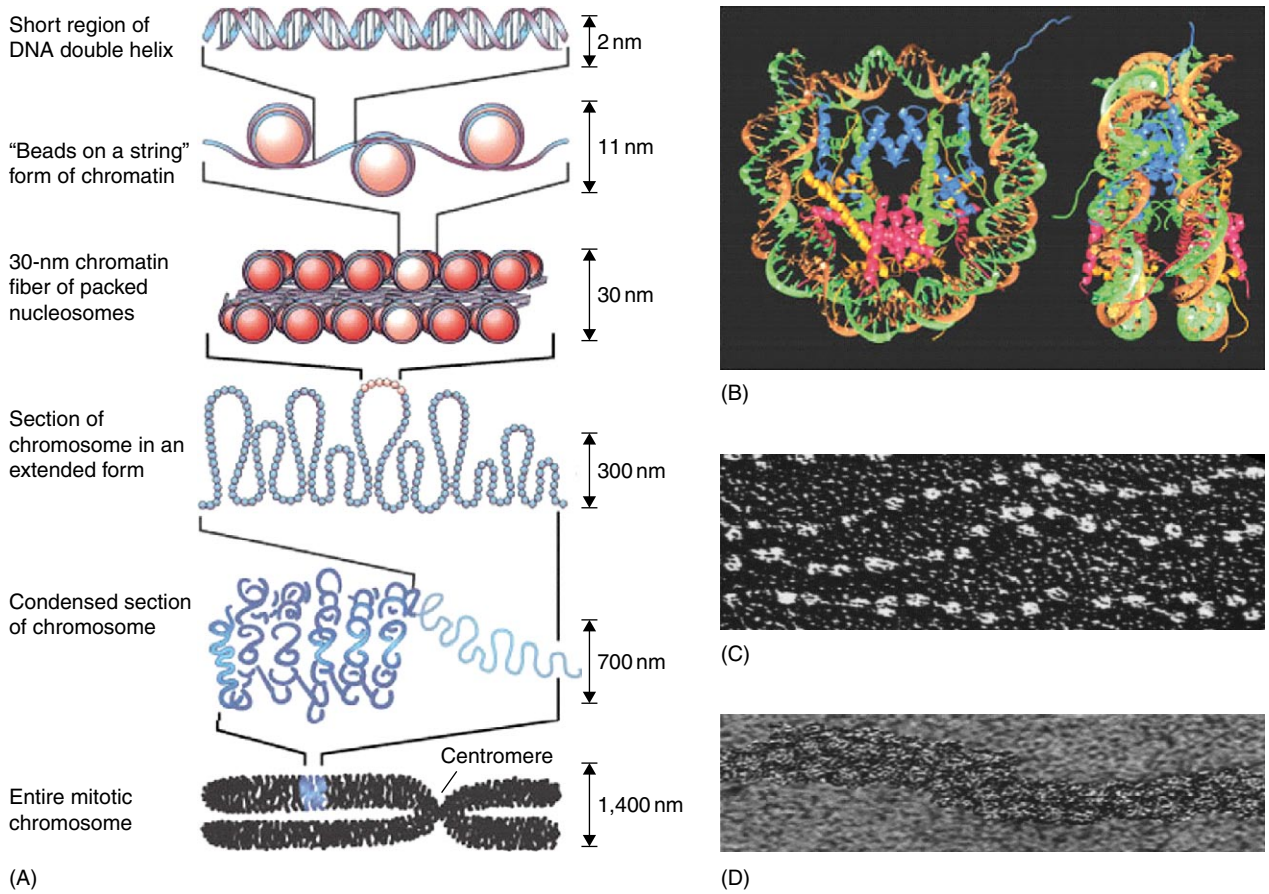


FIGURE 10-1 (A) Schematic of chromatin structure. 147-bp segments of DNA (top level) wrap 1.65 times around histone octamers to form nucleosomes (second level). Arrays of nucleosomes assemble into 30-nm solenoid structures (third level), considered to be the fundamental unit of higher-order chromatin. Extensive looping (fourth level) and condensation (fifth level) brought about by various proteins, including HMG members, results in the final configuration of the interphase nucleus, which undergoes further condensation to yield metaphase chromosome during mitosis (bottom panel). After returning to interphase, some regions of the genome remain highly condensed and are referred to as heterochromatin. Reproduced with permission from *Nature*. (B) X-ray crystal structure of nucleosome at 2.8 angstroms resolution viewed down the DNA superhelix axis (left) or orthogonal to the DNA superhelix axis. Molecular mass is 206 kDa and consists of DNA wrapped around the periphery of an H3 (blue)–H4 (green) tetramer flanked by two H2A (orange)–H2B (red) dimers. The structure of N-terminal histone tails cannot be determined in this crystal structure but protrude away and contact neighboring nuclesomes. Reproduced with permission from *Nature*. (C) Electron micrograph of nucleosomes as beads on a string as depicted in second level of part A. (D) Electron micrograph of 30-nm solenoid structure as depicted in third level of part A.

dogs that have survived, cloned mice are known to have impaired immune systems, become obese, and die prematurely. When cloned obese mice are bred, their progeny are not prone to obesity, indicating that this is an epigenetic defect that can be corrected by transmitting the genome through the germline to the next generation.

Unlike Cc, where XCI occurred normally, the ailments and lethality that occur in most clones are likely due to epigenetic defects where the donor cell nucleus retains some of its DNA methylation and other chromatin characteristics as a form of epigenetic memory, rather than being completely reprogrammed in the reconstructed oocyte. As a result, some donor cell-specific genes continue to be expressed inappropriately, and the *Oct4* pluripotency gene is often misexpressed, which might account for the observed periimplantation lethality. Aberrant DNA methylation and gene expression also probably explains *in utero* and post-natal lethality. Based on gene expression profiling experiments, it has been reported that 4–5% of all genes and 30–50% of imprinted genes are deregulated in the placentas from newborn mice conceived by SCNT, compared to mice conceived naturally.

Transcription Factors and Chromatin-Modifying Factors to Improve the Efficiency of Nuclear Reprogramming for Regenerative Medicine

Despite the low success rate, it is remarkable that cloning is ever able to work. After a nucleus from a somatic cell is transferred into oocyte cytoplasm, the chromatin structure must undergo rapid and extensive reprogramming to facilitate a genome-wide expression profile similar to an

early-stage, pre-implantation embryo following zygotic genome activation (ZGA). These changes must occur within minutes or hours in a SCNT experiment, whereas nuclear reprogramming takes place in the germline during spermatogenesis and oogenesis over weeks or months. Therefore, incomplete or aberrant nuclear reprogramming in clones is not unexpected.

Furthermore, nuclei from differentiated cells should require more extensive reprogramming and be less efficient donors than nuclei from relatively undifferentiated cells, and this prediction has been borne out experimentally (Hochedlinger and Jaenisch, 2006). The percentage of mouse clones that developed to term with nuclei from blastomeres of two-, four-, and eight-cell embryos decreased from 22% to 14% to 8%, respectively. Survival decreased to between 2–11% for embryonic stem (ES) cells and 1–3% for somatic cells from adults. This reduction in competency of nuclei from blastomeres occurs slightly later in cattle, which reflects the fact that ZGA occurs at the morula stage, as opposed to the two-cell stage. As a result, there is more time and cell cycles for transferred bovine nuclei to respond to signals from the oocyte cytoplasm by making wholesale changes in the chromatin structure before transcription is initiated.

Nuclear reprogramming is accompanied by the loss of many proteins from the donor nucleus, and the acquisition of many other proteins from the oocyte cytoplasm. For example, when nuclei from tadpole-derived cells are introduced into enucleated *Xenopus* eggs, more than 90% of TATA-binding protein is rapidly lost from the nuclear matrix, which is mediated by the ISWI chromatin-remodeling complex acting in an ATP-dependent manner, and there is a concomitant appearance of other proteins. Many other changes of this sort will undoubtedly require chromatin-modifying factors instead of occurring passively. For instance, a maternal-effect mutation of the BRG1 ATPase subunit of SWI/SNF-related chromatin-remodeling complexes in mouse oocytes results in defective ZGA and two-cell arrest. Considering that ZGA and SCNT are similar nuclear reprogramming events, maternal BRG1 might be required for SCNT to be successful.

As stem cells undergo cellular differentiation, sequence-specific transcription factors and chromatin-modifying factors interact to establish lineage-specific gene expression profiles that govern cell fate decisions. Once these transcription profiles and cell fates have been established, however, they can be maintained in the absence of sequence-specific transcription factors by chromatin-modifying factors, in some cases as a form of cellular memory. Therefore, it will be crucial to identify these factors and elucidate the corresponding molecular mechanisms. Not only will this be important from a basic research perspective, but it also has important implications for stem cell transplantation procedures and nuclear reprogramming in the clinical setting. For example, Sonic hedgehog and other factors have been used to differentiate ES cells into motor neurons, and dopaminergic neurons that function *in vivo*.

Conversely, it should be possible to coax a differentiated cell in the opposite direction into a less-differentiated stem or progenitor cell, considering the reversible nature of chromatin structure. In fact, a seminal 2006 study demonstrated it is possible to convert mouse fibroblasts into induced pluripotent stem (iPS) cells using several transcription factors (Oct4, Sox2, c-Myc, and Klf4). Mouse iPS cells share many characteristics with ES cells, including the ability to be transmitted through the germline of chimeras, and have been used to correct sickle cell anemia in a pre-clinical mouse model. More recently, several groups have used various combinations of transcription factors to convert human fibroblasts into iPS cells, and this approach has more potential in regenerative medicine than SCNT/therapeutic cloning, because it obviates the need for oocyte donors as well as for other technical and political reasons. IPS cell derivation currently utilizes retroviral vectors and is relatively inefficient, but manipulating chromatin might enable a different gene delivery system to be utilized and/or enhance nuclear reprogramming efficiency, which would improve the clinical potential of iPS cells.

Coordinate Action of Chromatin-modifying Factors

Over the last few years, chromatin research has made tremendous progress by utilizing genetic, biochemical, and molecular approaches in a broad range of model organisms. An important theme that has emerged from these multidisciplinary efforts is that DNA methylation, histone modifications, and ATPase chromatin-remodeling complexes are functionally interdependent. It is becoming evident that these interactions also apply to DNA replication and repair during interphase of the cell cycle, chromosome segregation in mitosis, and recombination in meiosis. A second theme is that chromatin-modifying factors from each of these three general categories underlie some of the most well-studied epigenetic processes: position-effect variegation (PEV), X-chromosome inactivation (XCI), Polycomb group (PcG) silencing, and trithorax group (trxG) activation, and monoallelic expression in both genomic imprinting and allelic exclusion. Recent work has provided considerable insight into the molecular mechanisms and revealed striking similarities. A third theme is that non-coding RNAs (ncRNAs) play an important role in many of these processes by directing chromatin-modifying factors and epigenetic marks to specific loci. Based on a pilot study of the ENCODE project, where 1% of the human genome was annotated in great detail, the vast majority of the DNA sequence in the genome is transcribed, primarily as ncRNAs. Although some of these ncRNAs might be nonfunctional byproducts of transcriptional noise, it seems likely that a large number will be crucial for epigenetic transcriptional silencing in addition

to microRNA- and RISC-mediated post-transcriptional silencing.

In the sections that follow, we review what is currently known about DNA methylation, the histone code, and ATPase chromatin-remodeling complexes. Subsequently, we discuss the role of these factors in PEV, XCI, PcG silencing and trxG activation, and monoallelic expression.

CHROMATIN-MODIFYING FACTORS

DNA Methylation

Mammalian genomes are extensively methylated, particularly at cytosines of CpG dinucleotides, which are symmetrically methylated on complementary strands of DNA (Bird, 2002). Programmed changes in patterns of this cytosine methylation during embryogenesis suggest that methylation may play an important role in cell fate specifications (Figure 10-2). Shortly after fertilization, the male pronucleus is rapidly and actively demethylated. Interestingly, the female pronucleus is resistant to this active demethylation, perhaps because the paternal genome only becomes accessible to putative demethylases during the protamine-to-histone conversion. From the two-cell stage to the blastocyst stage, DNA methyltransferases are largely excluded from nuclei, and both the paternal and maternal genomes undergo passive demethylation, such that the genome of implantation stage embryos is globally demethylated. Important exceptions are imprinted genes and certain retroviral-like elements, which maintain their methylation status during cleavage divisions. After implantation, cells of the mouse embryo proper are remethylated. In contrast, mouse extraembryonic cells experience only modest remethylation, suggesting that DNA methylation plays a less prominent role in these tissues.

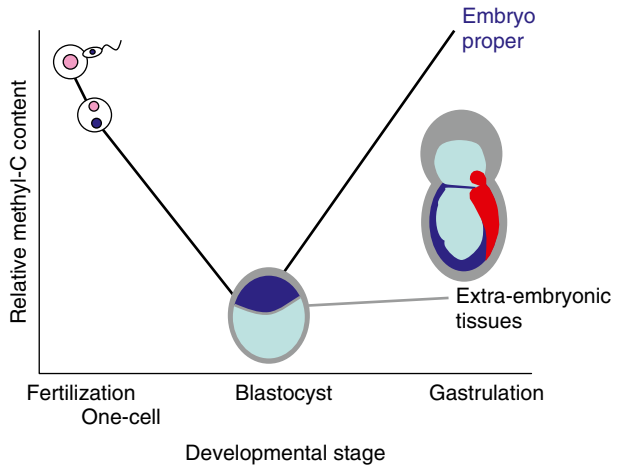


FIGURE 10-2 Dynamic changes in global DNA methylation during mouse embryogenesis. At fertilization, both gametes are hypermethylated. Shortly after fertilization, the male pronucleus (blue) is actively demethylated. Because DNA methyltransferases are initially excluded from the nucleus, passive demethylation occurs from the one-cell to blastocyst stages. After implantation, embryonic tissues undergo pronounced remethylation and level off at gastrulation. Remethylation is limited in extraembryonic tissues. Figure adapted from Li (2002).

Independent waves of demethylation and remethylation also occur in the germline. Primordial germ cells are actively demethylated in both sexes beginning around mouse embryonic day (E) 10.5 and finishing by E12.5. This demethylation erases the parental legacy of alleles and is presumably critical for imprinting. Subsequently, male germ cells are remethylated late in embryonic development during the leptotene stage of meiosis; female germ cells are remethylated postnatally, at around P6, during oocyte maturation. Recent work indicates that a class of ncRNAs similar to miRNAs, Piwi-interacting small RNAs (piRNAs), regulate CpG methylation of retrotransposans in the germline, which are known to be crucial for genomic stability. This finding, and other evidence, supports the notion that DNA methylation arose during evolution to silence retrovirus-like elements, particularly in the germline.

Several DNA methyltransferase enzymes have been identified and characterized. These enzymes are separated into two functional classes—the *de novo* and maintenance methyltransferases. DNMT3A and DNMT3B are *de novo* methyltransferases responsible for remethylation in post-implantation embryos and in germ cells. *De novo* methyltransferase activity is stimulated by DNMT3L, which resembles DNMT3A and DNMT3B but lacks the methyltransferase catalytic domain and lacks methyltransferase activity of its own. DNMT1 represents the second functional class of DNA methyltransferases. DNMT1 is a maintenance methyltransferase that prefers hemimethylated templates and is recruited to actively replicating DNA through an association with PCNA, the replication fork clamp. Once recruited, DNMT1 propagates the methylated state by copying methylation onto the nascent strand as a form of cellular memory.

DNA methylation is not inherently repressive, and the transcriptional consequences of DNA methylation appear to be mediated by interplay with other chromatin-modifying factors. Cytosine methylation alters the substrate presented to DNA binding proteins, imposing allosteric constraints that favor some interactions but block others. Methylated CpG dinucleotides in and around promoters often block transcriptional activator binding and recruit chromatin-modifying enzymes that promote higher-order, repressive chromatin conformations. Methyl-CpG binding proteins, such as MBD2 and MeCP2, can recruit histone deacetylase, histone methyltransferase, and ATP-dependent nucleosome remodeling complexes, which all work in concert to restrict access of the general transcription machinery to the DNA.

The importance of DNA methylation to cell physiology is perhaps best illustrated by the pathologies that result when the process of establishing and reading these marks go awry. Much as the exquisite fidelity of DNA replication is not absolute, somatic cells accumulate epimutations during

aging and carcinogenesis. For example, genome-wide CpG methylation profiles are nearly identical in monozygotic twins when they are children, but diverge markedly before they reach 50 years of age. In human tumors, the promoters of many tumor suppressor genes, including *RB* and *p16*, are often silenced by aberrant CpG methylation, resulting in the functional equivalence of loss of heterozygosity (LOH). These events probably outnumber point mutations deletions that occur somatically in tumors, and the relatively poor fidelity of CpG methylation may reflect the fact that it must be malleable to accommodate reprogramming in the germline. It is also possible for genetic lesions to affect CpG methylation, as mutations in *DNMT3B* cause a disease called ICF (Immunodeficiency, Centromeric region instability, and Facial abnormalities). In other diseases, methylation patterns are maintained more or less appropriately, but the cellular machinery responsible for recognizing those marks is disrupted. For instance, mutations of *MECP2* cause a neurological disorder known as Rett syndrome, and mice mutant for *Mbd2* exhibit behavioral defects, including compromised maternal nurturing of pups.

The Histone Code

DNA methylation is important for the proper expression of many genes and is perturbed in some diseases, but it cannot account for many other aspects of transcriptional regulation. First, most CpG islands are unmethylated, regardless of whether or not the associated gene is expressed. Second, many cell types are properly specified despite having globally hypomethylated genomes in *Dnmt1−/−* embryos. Lethality does not occur until closer to midgestation at E9.5–10.5. Third, and perhaps most importantly, the nematode *Caenorhabitis elegans* lacks detectable cytosine methylation, and the fruit fly *Drosophila melanogaster* has only trace levels, restricted to the earliest stages of embryogenesis. Thus, evolutionary conserved mechanisms other than DNA methylation must be crucial for transcriptional regulation.

In this regard, histones are intimately associated with DNA, and are among the most abundant and highly conserved proteins from yeast to human. Not only have the amino-acid sequences and three-dimensional structures been conserved to a remarkable extent, but so have a variety of post-translational modifications. These modifications are covalent and include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ADP ribosylation of specific residues of canonical histones (H2A, H2B, H3, and H4), usually in the N-terminal tails that protrude away from nucleosomes (Kouzarides, 2007). For example, Figure 10-3A shows the position and nature of some, but not all, modifications that occur on H3 N-terminal tails. Each of these modifications either promotes or inhibits transcription. The situation is complicated by the fact that some of the opposing modifications inhibit each other and are therefore mutually exclusive.

All of the covalent modifications listed above, and the interplay that occurs between them, are referred to as the histone code (Kouzarides, 2006). Enzymes such as histone acetyltransferases (HATs) and histone methyltransferases (HMTs) write the code by introducing specific modifications. These modifications then serve as docking sites for proteins that read and execute the code. For example, acetylated lysines can be recognized by the bromodomains of subunits of ATPase remodeling complexes to promote transcription. In contrast, methylated lysines can be bound by the chromodomain of HP1 or PcG proteins to facilitate higher-order chromatin structures that inhibit transcription. Proteins containing PHD fingers and tudor domains can also bind to methylated lysines.

Covalent modifications of histones must be labile so that the transcriptional status of a particular gene can be reversed. HATs are counteracted by histone deacetylases (HDACs), HMTs are counteracted by jumonji C (JmjC) domain demethylases, and kinases are counteracted by

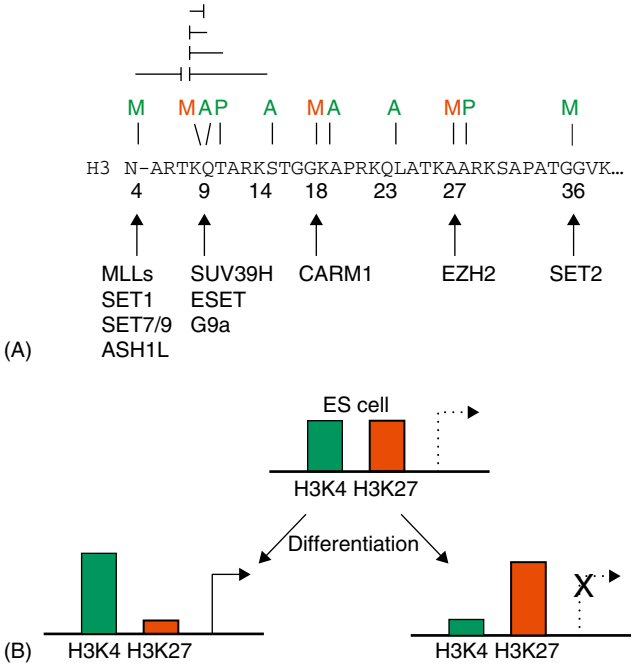


FIGURE 10-3 Covalent modifications of histones and bivalent domains. (A) Methylation (M), acetylation (A), and phosphorylation (P) of specific amino acids on the H3 N-terminal tail. Position of lysines (K) are indicated with numbers. All of the modifications either promote (green) or inhibit (red) transcription. K9 methylation is mutually exclusive with certain neighboring modifications (indicated above). Histone methyltranferases (HMTs) are shown below the residues they modify. This figure does not depict all H3 histone modifications or HMTs. (B) Schematic of a bivalent domain in ES cells. The promoter of a developmental regulatory gene is associated with active (H3K4, green) and repressive (H3K9, red) histone methylation in close proximity and is transcribed at low levels (dashed arrow) but poised for transcriptional upregulation or downregulation. The bivalent domain is resolved upon differentiation by JmjC domain histone demethylases and other factors. H3K27 methylation is decreased in cells that express the developmental regulator (arrow), whereas H3K4 methylation is decreased in cells that do not express the gene (obstructed arrow).

phosphatases. Most of the enzymes that either add or remove these covalent modifications do not function alone, but are present in multi-protein complexes. The other subunits in these complexes can increase the stability or catalytic activity of the enzymatic subunit or target it to specific genomic loci via protein–protein interactions with sequence-specific transcription factors.

Although activating and repressive histone modifications are usually not found in close proximity, H3K4 methylation (active) and H3K27 methylation (repressive) are juxtaposed in promoters of some developmental regulatory genes in ES cells (Figure 10-3B). These regions are referred to as bivalent domains and exhibit low-level transcription in ES cells, but are poised for high-level transcription depending on the course of differentiation. As ES cells differentiate into specific cell lineages, these bivalent domains are resolved through the action of JmjC demethylases and other chromatin-modifying factors. As a result, either H3K4 methylation persists and transcripton is increased, or H3K27 methylation persists and transcription is decreased.

Considerable interplay and a two-way flow of epigenetic information also occur between histone modifications and DNA methylation. For example, just as DNA methylation can influence histone deacetylation, histone methylation can influence DNA methylation. For example, genetic screens have uncovered mutations in HMTs that affect DNA methylation.

ATPASE CHROMATIN-REMODELING COMPLEXES

In addition to enzymes that covalently modify histones, there is a second evolutionarily conserved mechanism that modulates chromatin structure carried out by SWI/SNF and other ATPase chromatin-remodeling complexes (de la Serna *et al.*, 2006). SWI/SNF does not possess significant DNA binding ability of its own, but is recruited to promoters by sequence-specific transcription factors. The energy derived from ATP hydrolysis allows the complex to alter the conformation and position of nucleosomes. DNA-histone contacts are broken, and histone octamers can be slid several hundred base pairs upstream or downstream. As a result, a core promoter can be made nucleosome free and accessible to the RNA Polymerase II holoenzyme so transcription can be initiated. However, SWI/SNF can be recruited to other loci by transcriptional repressors and have the opposite effect by inhibiting transcription.

SWI/SNF complexes were first identified in yeast based on mutations that confer mating type switching or sucrose nonfermentation phenotypes. However, they are conserved in metazoans and have been well-characterized in *Drosophila*, mice, and humans. Due to duplication and divergence events during vertebrate evolution, mammals have an increased number of subunits. There are two subfamilies of mammalian complexes, BAF and PBAF, which have considerable overlap but differ for a few key subunits including the catalytic subunit (BRG1 or BRM for BAF, but only BRG1 for PBAF). Both BAF and PBAF complexes contain core subunits, but also exhibit further diversity by containing different accessory subunits in various tissues or cell types. For example, it was recently demonstrated that BAFs 45a and 53a are present in neuronal stem/progenitor cells undergoing proliferation, but are replaced by BAFs 45b/c and 53b as they differentiate into mature neurons and exit the cell cycle. This swap is functionally important because preventing it from occurring impairs differentiation. Mammalian BAF and PBAF complexes play numerous other roles in development and the prevention of diseases, including cancer (de la Serna *et al.*, 2006).

Additional chromatin-remodeling complexes have been characterized that are distinct from SWI/SNF, although each one has a catalytic subunit with sequence similarity to the DNA-dependent ATPase domain of SWI2/SNF2 (de la Serna *et al.*, 2006). ACF/CHRAC and NURF complexes utilize SNF2H or SNF2L and are similar to yeast ISWI, NuRD complexes utilize Mi-2/CHD subunits, while INO80 and SWR1-related complexes utilize INO80 and P400 with split ATPase domains. Furthermore, based on genome sequencing projects, mammals have 29 SWI2/SNF2-related genes, which probably encode catalytic subunits of novel chromatin-remodeling complexes that await identification and purification.

It is not clear why such diversity of ATPase chromatin-remodeling complexes has been selected for during evolution, but there are numerous possibilities. Some complexes may modulate higher-order chromatin structure, whereas other complexes might act on nucleosome arrays. Different complexes could act at distinct promoters or overlap at a large subset of loci. It is also possible that two complexes perform fundamentally different tasks at the same promoter to influence transcriptional initiation, or one could act downstream to influence transcriptional elongation. Precedent for this sort of division of labor comes from evidence that CHD1-containing complexes promote transcriptional elongation. In addition, a number of these complexes can replace canonical histones with variants, such as is the case for SWR1 and H2AZ or NURF and H3.3. Finally, some of these complexes are known to perform functions unrelated to transcription, such as regulation of DNA repair, replication, or chromatid/chromosome segregation.

There is a lot of interplay between ATPase chromatin-remodeling complexes and enzymes that covalently modify histones. For example, SWI/SNF-related complexes have been shown to work in concert with HATs. Sequence-specific transcription factors can recruit HAT complexes to promoters to acetylate histone tails. Acetylated lysine residues can then serve as docking sites for bromodomains of SWI2/SNF2/BRG1/BRM catalytic subunits, resulting in

increased affinity of SWI/SNF complexes for their chromatin targets. At other loci or at different stages of the cell cycle (e.g., late mitosis), the order is reversed, with SWI/SNF-related complexes recruited first and HAT complexes second. In addition, SWI/SNF-related complexes have been associated with HMTs, and NuRD complexes include HDAC subunits. There is also evidence for interplay with DNA methylation. Mutation of the BRG1 catalytic subunit results in hypermethylation of the β-globin locus, and mutations of a couple of putative catalytic subunits (LSH1 and ATRX) result in more widespread alterations in DNA methylation. Evidence also comes from methyl-binding proteins; MECP2 has been associated with SWI/SNF-related complexes, and MBD3 is an integral component of NuRD.

EPIGENETIC PROCESSES

Position-effect Variegation

Shortly after discovering the mutagenic properties of x-irradiation, Muller described the isolation of several radiation-induced mutations of the *D. melanogaster* white (*w*) eye-color gene in 1930. Interestingly, some mutant lines had eyes that contained patches of wild-type cells with red pigmentation, intermingled with patches of mutant cells lacking pigmentation and appearing white. Six years later it was demonstrated that the x-rays induced a chromosomal rearrangement in each mutant line, such as an inversion in $In(1)w^{m4}$ (abbreviated w^{m4} for white mottled 4), that resulted in pericentric heterochromatin being juxtaposed with the *w* locus. The regulatory elements and coding sequence of the *w* gene were not perturbed in any of these mutants, but heterochromatin would spread across the breakpoint and silence transcription in a subset of eye progenitor cells during development. In contrast, the heterochomatin would not spread far enough to reach the *w* gene in other eye progenitor cells, and it would be transcribed at wild-type levels. Clonal expansion of these cell populations subsequently produced patches of white and red eye color, respectively. This silencing process must be stochastic, because even genetically identical flies exhibit unique patterns of white and red coloration. This epigenetic process, which has been documented at other loci in *D. melanogaster* and in other organisms, is called position-effect variegation (PEV).

Because w^{m4} is such a visual example of PEV, it served as the foundation of genetic screens to identify genes that modify the extent of heterochromatin spreading in PEV. *E*(*var*) mutations were recovered that enhance PEV, resulting in increased heterochomatin spreading, and cause the *w* gene to be silenced in a greater percentage of eye cells. Therefore, *E*(*var*) mutants have eyes that are more white than typical w^{m4} flies. In contrast, *Su*(*var*) mutations were recovered that suppress PEV, inhibiting the spread of heterochromatin, and cause the *w* gene to be transcribed in a greater percentage of eye cells. These mutants have eyes that are redder than w^{m4} and therefore more closely resemble wild type. In these screens, most of the *E*(*var*)*s* and *Su*(*var*)*s* were isolated as dominant mutations. When homozygosed, more than half of these mutations are lethal, indicating that the majority of the corresponding gene products are essential.

Chromatin and Molecular Basis of PEV

Although *E*(*var*) and *Su*(*var*) gene products are not strictly defined by conserved features, most that have been characterized at the molecular level contain domains or motifs present in PcG, trxG, or other chromatin-modifying factors. The connection between PEV and chromatin has also been strengthened by observations that histone, HAT, HDAC, PcG, and trxG mutations can behave as *E*(*var*)*s* or *Su*(*var*)*s*. On a mechanistic level, the most significant progress has come from the analysis of *Suv39h* and *HP1*, which are mammalian orthologs of *D. melanogaster Su*(*var*)*3–9* and *Su*(*var*)*2–5* (which encodes heterochromatin protein 1 or HP1), respectively (Richards and Elgin, 2002). SUV39H methylates H3K9 in pericentric regions of the genome, and this covalent modification serves as a docking site for the chromodomain of HP1 (Figure 10-4A). HP1 is a structural component of heterochromatin, and, once situated at a particular H3K9 residue, it is thought to utilize a second domain, called the chromo shadow domain, to directly or indirectly bind another SUV39H molecule. This recruitment step results in H3K9 methylation of an adjacent nucleosome and is followed by more HP1 binding, thereby creating a feedback loop enabling heterochromatin to propagate over Mb intervals.

Insulators and boundary elements prevent pericentric heterochromatin from spreading distally down the entire chromosome and silencing every gene in its path. These elements are associated with elevated levels of histone acetylation, which may prevent SUV39H recruitment and H3K9 methylation, as well histone variants such as H2AZ and H3.3. The zinc-finger transcription factor CTCF is also localized to insulators and boundary elements and recruits cohesin to partition the genome into chromosomal domains.

RNAi and Sequence Specificity of PEV

SUV39H does not exhibit any specificity other than the fact that it methylates H3K9, so what directs its activity to histone tails located in pericentric chromosomal regions, known to be particularly rich in heterochromatin, but not other regions of the genome? Work from the *Schizosaccharomyces pombe* counterparts of *Su*(*var*)*3–9* (*Clr4*) and *HP1* (*Swi6*) suggest that an RNAi-like mechanism is crucial (Richards and Elgin, 2002) (Figure 10-4B). In wild-type fission yeast, DNA repeats that are highly enriched in pericentric regions are transcribed in a

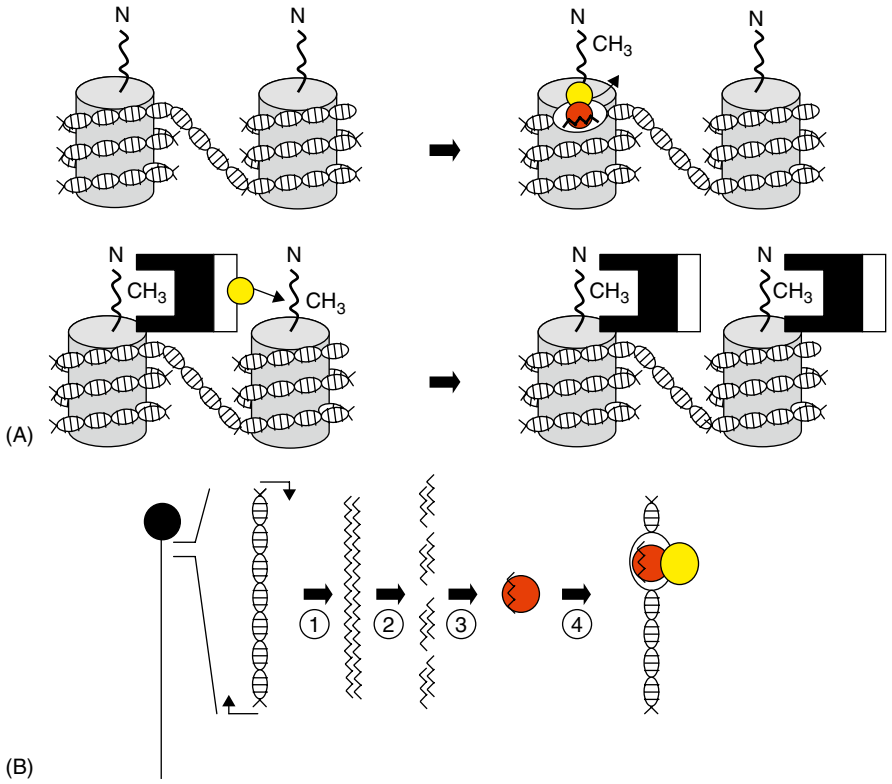


FIGURE 10-4 Specificity and propagation of epigenetic marks in PEV. (A) Specificity: DNA repeats in pericentric region are transcribed in a bi-directional manner (1). Double-stranded RNAs are cleaved by dicer (2) and processed by RNAi-like machinery (3) to yield RNA oligonucleotides that associate with argonaute and other proteins to form RNA-induced transcriptional silencing (RITS) complexes (red circle). RNA oligonucleotides are thought to hybridize to nascent transcripts at pericentric repeat that served as their template (4) and directly or indirectly recruit Clr4 histone methyltransferase (yellow circle). (B) Propagation: Clr4 (yellow circle) methylates H3K9 (CH3) (upper right panel), which serves as a docking site for the chromodomain of Swi6 (black part of box) (lower left panel). The chromo shadow domain of Swi6 (white part of box) is thought to directly or indirectly recruit more Clr4, which methylates H3K9 on adjacent nucleosome (barrel) (lower left). Recruitment of more Swi6 to nascent H3K9 methyl group enables next nucleosome to be modified (lower right panel).

bi-directional manner to produce 1.4- and 2.4-kbp double-stranded ncRNAs that are processed by dicer into approximately 22 nucleotide RNAs. Similar to short interfering RNAs (siRNAs) or micro-RNAs (miRNAs), these RNA oligonucleotides physically associate with argonaute and other proteins to comprise RNA-induced transcriptional silencing (RITS) complexes. However, unlike conventional miRNAs and RISC (RNA-induced silencing complexes), which move to the cytoplasm to bind and degrade cognate mRNAs, the heterochromatin-derived RITS complexes remain in the nucleus and recruit Clr4 to nascent transcripts at pericentric repeats, which methylate H3K9 and enable Swi6 binding (Figure 10-4B).

The studies described above were initially performed in *S. pombe* because the genes encoding Clr4, Swi6, and the RNAi machinery exist as singletons and were amenable to genetic screens. However, there is mounting evidence that RNAi-like directed changes in chromatin structure exist in other organisms and are not restricted to pericentric heterochromatin. Repeat-induced gene silencing of transgene concatamers is thought to involve dsRNAs (due to bidirectional transcription) targeting histone modifications and DNA methylation in *Drosophila* and mice, respectively. Co-suppression is also thought to involve dsRNAs and requires RNAi machinery and the PcG. More recently, an endogenous antisense transcript at the *p15* tumor suppressor locus that is up-regulated in leukemias was shown to silence *p15 via* H3K9 methylation and CpG methylation. A deletion immediately downstream of the human α globin locus, which does not ablate any α globin regulatory elements, removes the 3′ end (including the transcriptional stop signal) of a ubiquitously expressed gene transcribed in the opposite orientation. Continued transcription into the α globin locus results in an α2 antisense transcript, and formation of an aberrant dsRNA that causes abnormal CpG methylation and transcriptional silencing of the α2 promoter. Based on the conclusion of the ENCODE pilot project that there is a preponderance of ncRNAs in the mammalian genome, it seems likely that ncRNAs will provide sequence specificity to chromatin-modifying factors at many loci, and probably regulate many aspects of development and disease prevention.

X-CHROMOSOME INACTIVATION

XCI can be broken down into three general steps (Plath *et al.*, 2002): (1) counting, where the X:autosome ratio is determined in diploid cells so that every X chromosome except one will be inactivated (the n-1 rule); (2) choice, where either the maternal or paternal X is selected stochastically in the early post-implantation embryo or in ES cells upon differentiation; (3) the actual process of inactivation, which involves the X inactivation center (*Xic*) and is triggered by transcription of a large (17-kb) ncRNA called*Xist* from the future inactive X (Xi) that coats the Xi and directs a series of repressive epigenetic marks.

Xist and Heterochromatin Assembly

Prior to XCI, both X chromosomes are active (Xa), and transient pairing of the two X chromosomes at the *Xic* might play a role in choice. At this stage, *Xist* is transcribed from both X chromosomes at a low level. In mice, another large (40-kb) ncRNA that is transcribed in the opposite orientation and partially overlaps with *Xist*, called *Tsix*, is also expressed from both X chromosomes at modest to high levels. As XCI is initiated, *Xist* is up-regulated on the Xi and its ncRNA coats the Xi, but it is down-regulated on the Xa. The *Tsix* antisense transcript is expressed in a reciprocal manner as it is down-regulated on the Xi but persists on the Xa. Although *Xist–Tsix* dsRNAs have been detected recently, it is unclear whether *Tsix* blocks *Xist* transcription from the Xa using an RNAi-like strategy similar to that discussed for PEV, or using an unrelated mechanism. It also is not clear whether *Tsix* functions in a similar manner in humans and other mammals.

As XCI proceeds, the *Xist* ncRNA spreads across most of the Xi, but not the pericentric or pseudoautosomal regions, and a conserved repeat sequence near the 5′ end mediates silencing by recruiting chromatin-modifying factors (Plath *et al.*, 2002). Analysis of mouse ES cells as they undergo differentiation has identified a sequence of epigenetic events that initiate and maintain XCI (Figure 10-5). Histones associated with the Xi are hypoacetylated, hypomethylated at H3K4, and hypermethylated at H3K9 and H3K27. All of these marks are repressive, and H3K9 and H3K27 methylation are enriched at defined, nonoverlapping domains across the Xi in human cultured cells. The H3K9 methylated domains are associated with HP1 (analogous to PEV) and H4K20 methylation, whereas H3K27 methylated domains are associated with the macroH2A histone variant relatively late in the XCI process.

The PcG plays an important role in XCI because the EZH2 catalytic subunit of Polycomb repressive complex 2 (PRC2) methylates H3K27 on the Xi, which is subsequently bound by PRC1. The Ring1A/B components of PRC1 are E3 ligases that monoubiquitylate H2A at K119. Histone deacetylation continues after the action of PRC1

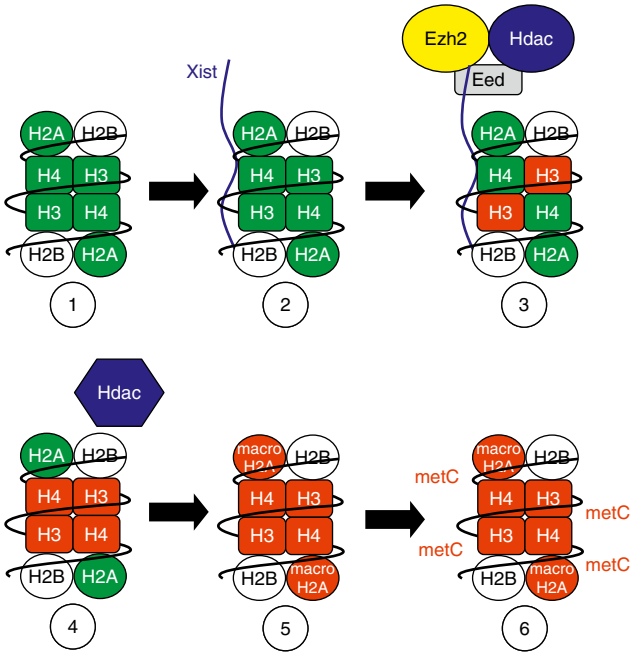


FIGURE 10-5 Sequential epigenetic marks during X-chromosome inactivation. For all histones (H2A, H2B, H3, and H4), green represents covalent modifications or variants associated with transcriptional activity, whereas red represents transcriptional repression. H2B is white because no covalent modifications or variants are known to be enriched on the inactive X chromosome (Xi). (1) Initially, both X chromosomes are H3 and H4 hyperacetylated, H3K4 methylated, and DNA hypomethylated. (2) *Xist* coats one of the two X chromosomes, which is destined to be the Xi, as the first step of the inactivation process. (3) Next, HDACs and EZH2/EED (components of Polycomb repressive complex 2) are recruited and mediate H3 deacetylation and H3K27 methylation, respectively. Concomitant with these modifications, H3K4 is demethylated. Subsequently, the Ring1A/B components of Polycomb repressive complex 1 monoubiquitylate H2A, H4 is deacetylated (4), and macroH2A replaces H2A in many nucleosomes on the Xi (5). (6) Finally, CpGs on the Xi become methylated.

and PRC2, and CpG methylation of the Xi is a very late event involved in maintenance of XCI. All of these epigenetic modifications result in the Xi being cytologically distinguishable as condensed heterochromatin (the Barr body in humans) at the nuclear periphery that replicates late during S phase. Outside of the pseudoautosomal region (PAR), transcriptional activity of the Xi is very low compared to the Xa, but transcription is not completely abolished because some genes escape XCI (see below).

Escape from XCI

A relatively large number of genes on the Xi, estimated to be ~15% of the total, escape XCI. These loci contain epigenetic marks enriched on the Xa (e.g., histone hyperacetylation and H3K4 methylation), are biallelically expressed, and replicate synchronously with the corresponding Xa alleles, indicating that they escape all aspects of XCI. Many of these genes reside in the PAR that carries

functional homologs on the Y chromosome. Thus, these genes are essentially diploid in both males and females, precluding any need for dosage compensation. Other genes escape XCI, despite lacking homologs on the Y chromosome, and should be less sensitive to dosage as a result. Many of these genes appear to have been acquired by the X chromosome after the mammalian sex chromosomes diverged and the X chromosome expanded due to translocations from autosomes (X-added regions) while the Y chromosome degenerated. Experiments in the mouse suggest that many of these genes undergo XCI initially, but are progressively reactivated as development proceeds. It is possible that LINE elements stabilize or maintain XCI, perhaps via CpG methylation, and genes that escape XCI might have a relatively low density of these repetitive elements in their vicinity. Similarly, it is possible that insulators or boundary elements prevent certain repressive chromatin marks from spreading into genes that escape XCI, leading to a silenced state that is less stable.

Reactivation and Imprinted XCI

Although XCI is stable in somatic cells, the Xi is reactivated in primordial germ cells during development of the germline. Following fertilization, XCI is re-established in the early mouse embryo within the first few cell divisions, but in an imprinted manner instead of stochastically. *Xist* is transcribed from the paternal X (Xp) chromosome, but not the maternal X (Xm) chromosome. *Xist* ncRNA spreads from the *Xic* along the rest of the Xp to inactivate it. At the blastocyst stage, imprinted XCI is maintained in the trophectoderm and primitive endoderm, which give rise to extraembryonic tissues, such as the placenta. In contrast, imprinted XCI is not maintained in the inner cell mass (which ES cells are derived from), which give rise to all of the cells of the embryo proper (Plath *et al.*, 2002). For reasons that are not yet clear, the Xp is reactivated and both X chromosomes are expressed in the epiblast until stochastic XCI occurs around the time of gastrulation. Interestingly, imprinted XCI might represent the ancestral form of XCI because it occurs in marsupials, but stochastic XCI does not.

Polycomb and Trithorax Groups

The Genetics of Polycomb Group (PcG)-mediated Cellular Memory

Genetic screens in *Drosophila* identified many PcG genes required for the proper expression of homeotic genes (Schwartz and Pirrotta, 2007). Whereas gap and pair-rule gene products are required to establish homeotic gene expression, PcG factors are required for maintenance. Not unexpectedly, these distinct classes of regulatory factors act in concert. Gap and pair-rule genes encode DNA-binding factors that bind to enhancers and promoters of homeotic genes in the *Antennapedia* (ANT-C) and *bithorax* (BX-C) complexes, and, in so doing, directly activate or repress transcription. However, these genes are transiently expressed and the corresponding gene products decay by mid-embryogenesis, yet homeotic gene expression is properly maintained throughout the remainder of development. Some gap and pair-rule proteins that repress transcription directly or indirectly recruit PcG factors before decaying. These PcG factors assemble into several complexes and alter chromatin structure. In so doing, the PcG complexes act as a form of cellular memory to keep inactive homeotic genes in a permanent "off" state over the course of many cell divisions. This regulation is crucial for proper anterior–posterior (A–P) patterning because homeotic genes, which are expressed in stem cell populations in the imaginal discs, control cell fate decisions in the head, the three thorax segments, and the eight abdominal segments.

Accordingly, PcG mutants exhibit ectopic homeotic gene expression and A–P patterning defects. In fact, going back to the original genetic screens, many PcG mutants were isolated as heterozygotes that have two or three thorax number one segments (T1–T1–T3 or T1–T1–T1) instead of the normal T1–T2–T3 pattern. These T2–to–T1 and T3–to–T1 homeotic transformations are most evident in male flies because mating structures, called sex combs, which are normally found only on the first pair of legs emanating from T1, are found on the first two or all three pairs of legs. This observation accounts for the names of many PcG genes: Polycomb (*Pc*), extra sex combs (*esc*), additional sex combs (*Asx*), sex combs on the midleg (*Scm*), posterior sex combs (*Psc*), etc. Moreover, when many of these same mutations are made homozygous, homeotic gene expression is altered to a greater extent, homeotic transformations are more severe and widespread, expression of other types of genes is perturbed, and embryonic or larval lethality often result. In addition, robust genetic interactions among mutations in distinct PcG genes exacerbate these mutant phenotypes.

PcG Complexes Cause a Repressive Chromatin State to Silence Transcription

PcG proteins assemble into at least three multi-protein complexes that are recruited to genomic loci in an ordered manner (Schwartz and Pirrotta, 2007). The PhoRC complex contain PHO and PHOL (homologs of mammalian YY1), which are two of the few PcG proteins known to bind DNA directly, and this early-acting complex binds to regulatory sequences referred to as Polycomb response elements (PREs). PREs are not strictly defined by DNA sequence, and are not highly conserved, even among different species of *Drosophila*. However, they usually contain PHO and PHOL binding sites (GCCAT) and often contain binding sites for other PcG or PcG-related factors such as DSP, ZESTE, or GAGA. Although PREs exhibit poor sequence conservation, they mediate robust silencing when

incorporated into transgene reporter constructs such as the *w* eye-color gene, and at endogenous loci including the ANT-C and BX-C. PhoRC silences transcription by recruiting Polycomb repressive complex 2 (PRC2) followed by PRC1 to establish a repressive chromatin structure. The PRC2 complex contains E(Z) (a homolog of mammalian EZH2) and several other core subunits including ESC (mammalian ESC). E(Z) (as well as EZH2) has a SET lysine methyltransferase domain and methylates H3K27, which serves as a docking site for the chromodomain of Polycomb (PC) (a homolog of several CBX factors), which is the eponymous subunit and a member of PRC1 (Figure 10-6). The PRC2-PRC1 recruitment step is analogous to SUV39H/Clr4 and HP1/Swi6 in PEV, although the spreading is less extensive (~10–100 kb versus several Mb). Spreading might be less extensive because PC (CBXs) lacks a chromoshadow domain and cannot recruit E(Z) to neighboring nucleosomes as efficiently; alternatively, insulators block silencing when placed between a PRE and the promoter of a reporter gene, and there might be a higher density of insulators in euchromatin than pericentric heterochromatin. The PRC1 complex also includes dRing/SCE (a homolog of mammalian RING1a/b), which ubiquitylates H2A at K119. PRC2 and PRC1 function together to form a chromatin structure that is repressive to transcription, at least one PcG member can interact with PCNA to maintain the repressive state following DNA replication over the course of many cell divisions.

Mammalian PcG Complexes in Development and Disease

Mammalian PcG gene products are more numerous, due to duplication and divergence events during vertebrate evolution, but comprise similar complexes and perform similar roles in development. Null mutations of *Ezh2*, *Eed*, and *YY1*, all of which encode components of PRC2, result in early embryonic lethality. However, it is clear that the function of this complex is not restricted to early embryogenesis, but is instead involved in a variety of biological processes in many different tissues. An *Ezh2* conditional mutation in B cells demonstrated that the wild-type gene product is required for VDJ recombination. *Ezh2* is also overexpressed in prostate cancer and several other tumors, and, conversely, RNAi down-regulation of *Ezh2* inhibits the proliferation of prostate cancer cells *in vitro*. Although *Eed* null-mutants exhibit severe A–P patterning defects during gastrulation, they die before many *Hox* genes are expressed. However, an ENU-induced hypomorph mutant line misexpresses *Hox* genes and exhibits homeotic transformations. Placental

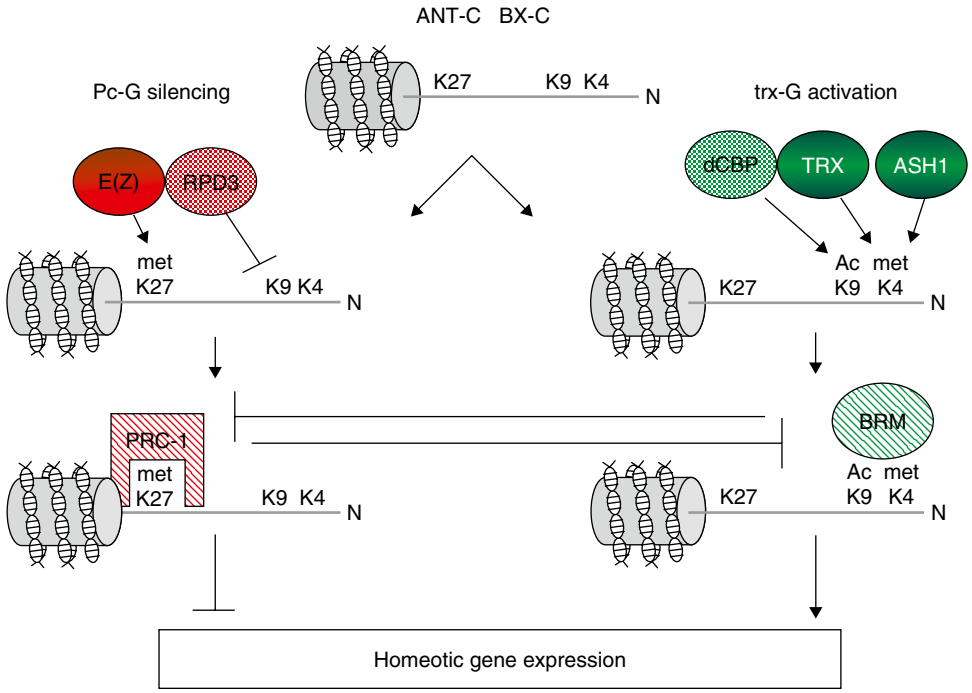


FIGURE 10-6 Homeotic gene regulation by Polycomb- and trithorax-groups. Schematic illustrating antagonistic roles of PcG silencing and trxG activation in homeotic gene regulation. A single nucleosome in the ANT-C or BX-C is shown with an H3 N-terminal tail projecting to the right (top panel). PcG silencing is initiated by the addition of repressive covalent modifications: E(Z) methylates (met) K27, and RPD3 deacetylates K9 (upper left). PRC1 complex binds met K27 (lower left). trx-G activation is initiated by the addition of other covalent modifications: TRX and ASH1 methylate (met) K4, and dCBP acetylates (Ac) K9 (upper right). A SWI/SNF-related BRM complex is recruited to K4-methylated, K9-acetylated nucleosomes (lower right). PRC1 and BRM recruitment are mutually exclusive (lower panels). Whereas PRC1 recruitment represses homeotic gene transcription, BRM promotes expression (bottom panel).

defects and T-cell lymphomas have also been reported. *Eed* also plays a role in XCI and genomic imprinting.

Null mutations of PcG genes that encode components of the mammalian PRC1 complex generally do not confer embryonic-lethal phenotypes. *MPc2/M33/Cbx2*, *Bmi1*, *Mel18*, and *Rae28/Mph1* homozygotes exhibit altered *Hox* gene expression and homeotic transformations, which are manifest by changes in vertebral identities, as well as cell proliferation defects in the hematopoietic system. One exception is *Ring1B/Rnf2*, but, although null-mutants die during gastrulation, hypomorphs are viable and do exhibit altered *Hox* gene expression and homeotic transformations. Of all these genes, *Bmi1* is particularly noteworthy because it inhibits the *Ink4a/p16* tumor suppressor locus and is required for continued proliferation of hematopoietic stem cells. It also regulates proliferation in more differentiated cells in several hematopoietic lineages. Gain-of-function mutations down-regulate both *Ink4a/Arf* gene products (the p16 and p19 cyclin-dependent kinase inhibitors) and cooperate with *c-Myc* in B- and T-cell lymphomas. Conversely, loss-of-function mutations lead to increased expression of p16 and p19 and decreased proliferation of erythrocytes, resulting in anemia. Moreover, this phenotype is suppressed by an *Ink4a* targeted mutation. *Bmi1* also plays a role in the self-renewal of neuronal stem cells, and has recently been shown to be a marker of intestinal stem cells.

Trithrax Group (trxG) Activation

Genetic screens in *Drosophila* identified loss-of-function mutations in *trithorax* (*trx*) and a number of other genes that suppress dominant *Pc* mutant phenotypes (Schwartz and Pirrotta, 2007). This t*rx* group (trxG) of genes also encodes proteins that have a variety of chromatin-related domains and motifs, some of which are shared with *Pc*G members, but have the opposite effect on transcription. For example, just like E(Z), TRX (a homolog of mammalian MLLs) also has a SET domain (which stands for *Su*(*var*)*3–9*, *E*(*z*), and *trx*) but methylates H3K4 instead of H3K27 and is involved in transcriptional activation of homeotic genes instead of transcriptional repression (Figure 10-6). Consequently, mutations in *trx* or other trxG genes result in decreased homeotic gene expression instead of ectopic expression. Of course, the fact that trxG and PcG counteract each other at the molecular level is not surprising, but expected, considering that the two groups have an antagonistic relationship at the genetic level.

Genetic and molecular interactions among other trxG genes, which encode various chromatin-modifying factors, are common and important at the mechanistic level. TRX physically binds to another HMT called ASH1, and both TRX and ASH1 bind to the dCBP histone acetyltransferase. TRX also binds to the SNR1 (a SNF5 homolog) subunit of SWI/SNF-related complexes. Several SWI/SNF subunits including SNR1, OSA (a homolog of mammalian BAF250), BRM (a homolog of mammalian BRM and BRG1), and MOIRA (a homolog of mammalian BAF170 and BAF155) were isolated as trxG members. SWI/SNF binding is reinforced by the bromodomain of BRM binding H3K9 acetylation (Figure 10-6).

Mammalian counterparts of trxG genes are important for many aspects of development, ranging from ZGA and implantation to *Hox* gene expression, erythropoiesis, and neural tube closure at midgestation, to postnatal fitness and cancer prevention in adults. With respect to *Hox* gene regulation, trxG and PcG factors counteract each other just as they do in *Drosophila*.

MONOALLELIC EXPRESSION: GENOMIC IMPRINTING AND ALLELIC EXCLUSION

Mammals are diploid for all autosomal loci, inheriting one copy of each gene from each parent. Presumably, both alleles of most genes are utilized. However, for a growing number of characterized genes, only one allele is transcribed per cell, a process termed monoallelic expression. For genes subject to monoallelic expression, each cell is functionally hemizygous, expressing either the maternally- or paternally-inherited allele, but not both.

Genomic Imprinting

The best-studied type of monoallelic expression is genomic imprinting, whereby genes are expressed according to their parental origin (Reik and Walter, 2001). For example, only maternally-inherited copies of *H19* are expressed (paternal alleles are silenced), and only paternally-inherited copies of *Igf2* are expressed. Such parent-of-origin expression for imprinted genes explains the inability of mammalian partheongenotes, gynegenotes, and androgenotes, as well as certain uniparental disomies, to develop to term. It also explains why inheritance of certain mutations from one parent does not confer a phenotype (because it was epigenetically silenced anyway), whereas inheriting the same mutation from the other parent results in a severe clinical syndrome (because the expressed allele is mutated and the wild-type allele is silenced). Prader-Willi syndrome and Angelman syndrome are particularly interesting, because they involve reciprocally imprinted genes on 15q11–q13.

To date, 15 clusters encompassing roughly 80 imprinted genes have been identified in the mouse (www.mgu.har.mrc.ac.uk/imprinting/all_impmaps.html). These clusters are generally associated with regions of DNA that are differentially methylated in sperm and oocytes. These differentially methylated regions (DMRs) are considered imprinting control regions (ICRs), because they appear to regulate entire imprinting clusters. Targeted deletion of ICRs leads

to loss of imprinting (biallelic expression or silencing) of all of the genes in the cluster. Additionally, *Dnmt1−/−* embryos exhibit loss of imprinting of almost all imprinted genes, and the progeny of both *Dnmt3L*, *Dnmt3a−/−*, and *Dnmt3b−/−* females exhibit loss of imprinting of maternally-imprinted genes. A conditional mutation of *Dnmt3a* in primordial germ cells also leads to a loss of imprinting (LOI). These findings have led to a general consensus that DNA methylation is a critical part of the biochemical imprint that distinguishes maternal and paternal alleles at imprinted loci.

In addition to CpG methylation, other epigenetic marks that distinguish parental alleles at imprinted loci have begun to emerge. Nucleosomes associated with active alleles tend to be hyperacetylated on H3 and H4 tails and methylated at H3K4. Conversely, histones associated with silenced alleles tend to be hypoacetylated and methylated at H3K9 and H3K27. The enzymes responsible for these modifications at imprinted loci remain largely undefined. Loss-of-function mutations in the PcG gene *Eed* lead to biallelic expression of several imprinted genes, including *Mash2* and *Cdkn1c* in the *Kcnq1* cluster on mouse chromsome 7. EED associates with EZH2, an HMT with activity directed against H3K27. Future work will need to address allele-specific association of the latter mark at imprinted loci. Interestingly, allele-specific DNA methylation remains intact at genes affected in *Eed−/−* embryos. This observation suggests that covalent histone modifications at imprinted loci are either parallel to, or downstream of, DNA methylation. Consistent with this model, MeCP2 recruits histone H3K9 histone methyltransferase activity to the maternally silenced allele of *H19*. This finding indicates that allele-specific DNA methylation may drive allele-specific transcriptionally repressive chromatin marks.

Similarities and Differences Among Imprinted Gene Clusters

Six imprinted clusters have been characterized in detail and share several features: (1) they are relatively large (100-kb to 3-Mb) and include reciprocally imprinted ncRNAs and mRNAs as well as non-imprinted mRNAs; (2) the imprint is established at the DMR of one gamete and maintained on one chromosome of diploid cells; (3) the DMR is close to the promoter of an imprinted ncRNA and positively regulates its expression and also regulates the expression of other imprinted genes in the cluster in *cis*; (4) *Dnmt* mutations perturb DMR function and lead to LOI.

However, there are also important differences among the six clusters that allow them to be placed into two general categories. The *Igf2/H19* and *Dlk1* clusters utilize a CTCF insulator mechanism (Figure 10-7A). The DMR is methylated paternally, and the imprinted ncRNA does not have a strong effect on silencing imprinted mRNAs in the cluster based on gene targeting experiments. These imprinted ncRNAs are maternally expressed, relatively small (several kb), and located downstream of the imprinted ncRNAs in the same transcriptional orientation. In contrast, the *Igf2r/Air*, *Kcnq1*, *Pws*, and *Gnas/Nespas* clusters utilize a large, antisense ncRNA mechanism (Figure 10-7B). The DMR is methylated maternally, and the imprinted ncRNA has a strong effect on silencing imprinted mRNAs in the cluster based on gene-targeting experiments. These imprinted ncRNAs are paternally expressed, large (>60-kb and possibly up to 1-Mb), and embedded in the intron(s) of an imprinted mRNA gene in an antisense orientation.

Allelic Exclusion

Imprinted genes are a subset of a much larger number of genes that are monoallelically expressed. For many monallelically expressed genes, the active allele is determined not by parent-of-origin as in imprinting, but by allelic exclusion, where a stochastic choice dictates one allele to be active and one to be silent. Our understanding of allelic exclusion lags behind our understanding of imprinting, because the mosaic nature of the process requires analyses to be performed at the level of single cells (i.e., expression analyses of a mosaic tissue will suggest biallelic expression). The technical obstacles to studying allelic exclusion have also made it difficult to estimate what fraction of the genome might be subject to such regulation. However, a recent study that utilized microarrays to detect single nucleotide polymorphisms (SNPs) in primary transcripts reported that ~9% of 3939 genes with detectable SNPs exhibited monoallelic expression in B lymphoblast clonal lines. Genes encoding transmembrane receptors were overrepresented. This study also revealed considerable epigenetic heterogeneity among these genes, as any given locus was monoallelically expressed in 20% of the clonal lines but biallelically expressed in the other 80%.

Most work on allelic exclusion has been performed on genes encoding odorant and pheromone receptors, which number just under 1000 in human and just over 1000 in mouse, and a number of immunological molecules, including the immunoglobulin loci and the T-cell receptor. Not only do these loci undergo allelic exclusion, but only a single gene is expressed per olfactory neuron or lymphocyte. Despite many similarities, SCNT experiments have uncovered a fundamental difference between the regulation of odorant receptor and immunoglobulin expression. When nuclei from olfactory neurons are used as the donor, cloned mice that survive express the entire repertoire of odorant receptors, and the one expressed by the donor cell nucleus is expressed in ~0.1% of the olfactory neurons. In contrast, when nuclei from B-cells are used as the donor, cloned mice that survive are considered monoclonal because every B-cell expresses the same immunoglobulin as the donor cell. This epigenetic versus genetic difference

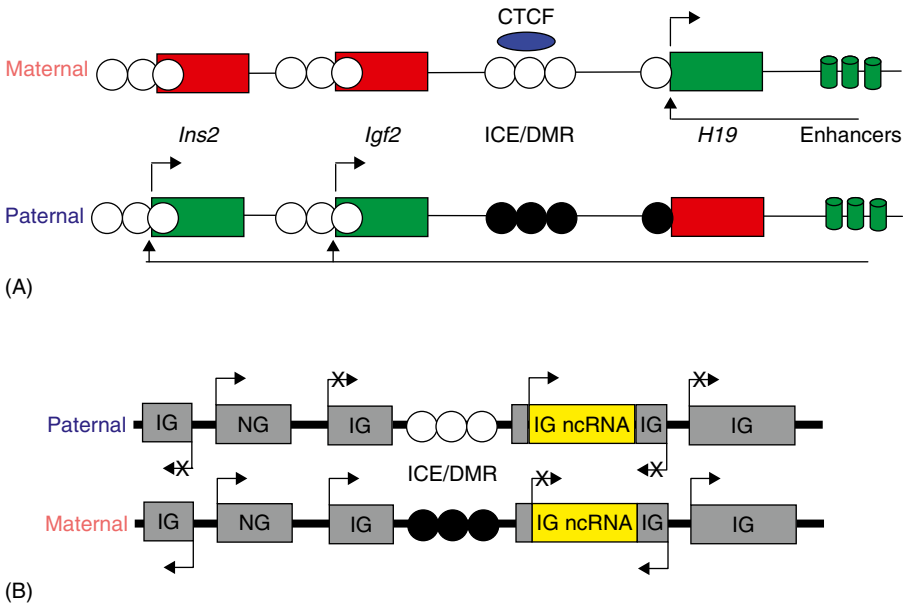


FIGURE 10-7 Imprinted gene clusters utilize two *cis*-acting mechansims. (A) CTCF insulator model exemplified by the Igf2/H19 cluster. A differentially methylated region (DMR) between Igf2 and H19 acts as an imprinting control element (ICE). Top: The DMR of the maternally-inherited allele is unmethylated (three white circles) and binds CTCF (blue oval), which blocks the association of *Igf2* with enhancers (three barrels) downstream of *H19*, leading to *Igf2* and *Ins2* silencing (red) in *cis*. Because the CTCF binding sites do not lie between *H19* and these enhancers, *H19* ncRNA is transcribed (arrow, green) from this chromosome. Bottom: The DMR of the paternal allele is methylated (three black circles) and cannot bind CTCF, which facilitates interaction between *Igf2*, *Ins2*, and the downstream enhancers, leading to *Igf2* and *Ins2* transcription (arrow, green). Conversely, *H19* promoter is methylated (1 black circle), recruits MeCP2, and is silenced (red). Current models postulate that *Igf2* and *Ins2* have greater affinity than *H19* for the downstream enhancers and therefore, in the absence of CTCF insulation, *Igf2* and *Ins2* outcompete *H19* for these enhancers. (B) Large ncRNA model. The DMR/ICE is in close proximity to the promoter of an imprinted gene transcribed into a large ncRNA (IG ncRNA, yellow box), which is embedded in one or more introns of an imprinted gene that it transcribed in the opposite orientation and codes for a protein (IG). Top: The DMR of the paternally-inherited allele is unmethylated (three white circles) and directs transcription of the IG ncRNA, which silences transcription of the other imprinted genes in the cluster (IG) in *cis*. This silencing may involve a dsRNA and RNAi-like mechanism (especially for the embedded IG) and/or involve the IG ncRNA spreading and coating the cluster in *cis* similar to *Xist* and XCI. It does not silence transcription of non-imprinted genes in the cluster (NG) or imprinted genes of this cluster on the maternally-inherited chromosome. Bottom: The DMR is methylated (three black circles) and the imprinted IG ncRNA embedded in the intron(s) (yellow box) is not expressed. As a result, there is no silencing of imprinted genes (IG) on non-imprinted genes (NG).

is presumably due to VDJ recombination at the immunoglobulin locus in B-cells.

For genes that undergo allelic exclusion, alleles are distinguished not only by expression state, but also by DNA methylation status, DNase I hypersensitivity, and replication asynchrony. Generally, active alleles are hypomethylated, DNaseI hyperaccessible, and early replicating, whereas silent alleles are hypermethylated, DNase I hypoaccessible, and late replicating. Replication asynchrony is established around implantation, long before antigen receptor rearrangement or the onset of odorant receptor expression, and roughly coincident with other stochastic epigenetic choices, including XCI. Also similar to XCI, the epigenetic states dictated by implantation-stage choices are propagated through mitoses to generate clonal populations of cells. Finally, allelic exclusion choice may not occur at the level of individual genes as anticipated, but at the level of entire chromosomes. The early replicating alleles of any two linked genes subject to allelic exclusion appear always to be on the same chromosome. Essentially, many or all chromosomes may be subject to processes mechanistically similar to XCI, but a much higher proportion of genes may escape on autosomes. Future work will need to extend the replication assays and determine whether transcriptionally active alleles are always on the same chromosome.

SUMMARY AND FUTURE DIRECTIONS

Drawing upon several models of epigenetic inheritance, much has been learned about chromatin-modifying factors and how they act and interact to regulate transcription. It is becoming increasingly clear that these factors and their corresponding epigenetic marks dictate developmental potentials. The present challenge is to understand better chromatin-remodeling mechanisms in cell fate decisions, cell proliferation and differentiation, and plasticity. In addition to providing considerable insight into many aspects of embryogenesis and postnatal development, this knowledge should prove valuable for improving the prospects of stem cell technologies in the clinical setting.

KEY WORDS

ATPase remodeling complexes Multi-subunit protein complexes that utilize a catalytic ATPase subunit to alter the conformation and structure of nucleosomes and modulate chromatin structure, and therefore transcription and other processes.

Chromatin The structure that the genome adopts as a result of DNA being physically associated with histones and non-histone proteins in the nucleus of eukaryotic cells.

DNA methylation The addition of a methyl group to specific nucleotides in genomic DNA, which in mammals occurs on cytosines almost always in the context of CpG dinucleotides.

Histone code The action and interaction of covalent histone modifications, which are enriched on N-terminal histone tails, to affect transcription and other biochemical processes.

Imprinting Monoallelic expression of a gene in a diploid cell based on maternal versus paternal inheritance.

Nucleosome The most fundamental unit of chromatin, consisting of 147-bp segments of DNA wrapped 1.65 times around histone octamers.

Pc-G and trx-G The polycomb and trithorax group genes encode multi-subunit complexes that counteract each other at the level of chromatin structure to maintain the expression of Hox genes and other genes in a silent state and active state, respectively.

Position-effect variegation A type of mosaic gene silencing where heterochromatin spreads from one genomic interval, such as pericentric repeats following their mislocalization following a chromosomal rearrangement, into flanking sequences to differential extents, resulting in a gene being silenced in a subset of cells.

X-chromosome inactivation Mammalian dosage compensation mechanism normally involving inactivation on one out of two X-chromosomes in females.

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Chapter 11

Cell Fusion and the Differentiated State

Penny Johnson and Peter W. Andrews

INTRODUCTION

Cell fusion recently surfaced as an issue in analyzing data from experiments designed to demonstrate the pluripotency of various adult stem cells. However, the phenomenon of spontaneous cell fusion has been known for a considerable time, and experimentally induced cell fusion has been widely used for years, for both genetic analysis and studies of the differentiated and determined states.

In studies made over 40 years ago, Sorieul and Ephrussi (1961) found that the co-culture of two mouse cell lines with differing marker chromosomes resulted in the appearance of a significant proportion (up to 10%) of spontaneously derived hybrid cells during about three months of continuous culture. Subsequently, it was shown that specific drug selection techniques could be used to isolate such hybrid cells and the hypoxanthine-aminopterin-thymidine (HAT) selection system to eliminate parental cells separately deficient in hypoxanthine phosphoribosyl transferase (HPRT), and thymidine kinase (TK) was introduced. Genetic complementation resulting in expression of both enzymes allowed for survival of the hybrids. It was then found that a substantial loss of human chromosomes occurs in spontaneous hybrids between diploid human fibroblasts and mouse L-cells, while the complete set of mouse chromosomes is retained. This phenomenon of specific chromosome loss, coupled with the subsequent discovery that cell fusion could be induced by inactivated Sendai virus, or by incubating cells in polyethylene glycol (PEG), provided a powerful new method for human gene mapping, which was widely used during the 1970s. Thus, by correlating the expression of human traits in such hybrids with the retention of particular human chromosomes, individual human genes could be located on specific chromosomes or even parts of chromosomes.

HYBRID CELLS AND DIFFERENTIATED PHENOTYPES

Simultaneously with the development of techniques for producing hybrid cells, John Gurdon and his colleagues demonstrated that a nucleus obtained from a differentiated cell of a tadpole, when transferred to an enucleated frog oocyte, supported the development of an adult frog. The general conclusion from these studies was that all differentiated cells retain full genetic potential for development and that the differentiated states of functionally distinct cell types must therefore arise from differential regulation of gene activity. What, then, would be the result of combining the genomes from different cell types in a single hybrid cell?

In 1965, Henry Harris had described the behavior of different nuclei in heterokaryons formed between various cell types. The most striking of these combinations was the fusion of HeLa cells with mature chicken erythrocytes in which, unlike mammalian erythrocytes, the chromatin is condensed and the nucleus is inactive. In the resulting heterokaryons, the chicken erythrocyte nuclei became active for both DNA and RNA synthesis and re-expressed chicken-specific genes. This result was confirmed, and shown to be the result of cytoplasmic factors. For example, chicken erythrocyte nuclei were found to be reactivated when introduced into enucleated cytoplasm. Furthermore, the reactivated chicken nucleus in cells reconstituted with fibroblast cytoplasm supported synthesis of chicken globin.

Attempts to discern general rules from the fusion of cells of different phenotypes, however, proved difficult. Despite the results with erythrocyte heterokaryons, an early conclusion was that the fusion of cells expressing two distinct states of differentiation frequently resulted in the loss of those differentiated functions in the hybrid cells. For example, hybrids formed between fibroblasts and melanomas did not produce melanin, and globin synthesis

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was not inducible in hybrids between fibroblasts and Friend erythroleukemia cells. On the other hand, hybrids between human leukocytes and mouse liver tumor cell lines sometimes expressed liver-specific proteins from the human genome contributed by the fibroblasts. Similarly, rat hepatoma–mouse lymphocyte or fibroblast hybrids often expressed mouse albumin. In other studies, hybrids between neuroblastoma cells, which exhibit a variety of neural features, and mouse L-cells, which are long-established, immortalized fibroblastoid cells, retained at least some of the neural features of the neuroblastoma parental cells, notably their electrical activity.

Apart from attempting to investigate the control of specific differentiated states, Harris and his colleagues, in particular, sought to use cell hybridization to establish the mode of genetic control of the transformed state of cancer cells, in particular whether the genetic changes that underlie the transformed state of tumor cells were the result of the loss or gain of gene function. In a conclusion that presaged the identification of tumor suppressor genes, they reported that malignancy acted as a recessive trait at the cellular level, as fusion of malignant cells with nonmalignant partners resulted in the formation of nonmalignant hybrid cells in which malignancy reappeared with subsequent chromosome loss. Clearly, such a result is not always the case, as might be inferred from our current knowledge of oncogenes, as well as tumor suppressor genes. Perhaps the most notable exception to this rule was the formation of immortal and tumorigenic "hybridomas" that produced monoclonal antibodies following the fusion of terminally differentiated plasma cells and a lymphoid cell line.

Generally, it seemed that hybrids of cells with distinct phenotypes did not express a hybrid phenotype. Rather, they tended to express genes associated with one or other of the parent cells, but not both. For example, mouse hepatoma–Friend erythroleukemia hybrids were described, which continued expressing liver functions, but in which globin expression was extinguished. Furthermore, in some cases, the gene expression typical of one parental phenotype was activated from the genome of the other parental cell. However, no clear rules emerged as to which phenotype would predominate.

The mechanisms by which one phenotype predominates over another in such hybrids largely remains poorly understood. In some cases, at least, the genome of one contributing nucleus retained the capacity for reactivation of its tissue-specific genes, even when extinguished in the initial hybrid cells. For example, in Chinese hamster fibroblast–rat hepatoma hybrids, rat liver functions were extinguished, only to reappear in some subclones on subsequent passage, possibly because of the loss of particular chromosomes. This result implies a stable modification to the genome responsible for the maintenance of its epigenetic state and not erased in hybrid cells. In other situations, experiments with phenomena such as imprinting and X-inactivation indicate that DNA methylation and histone acetylation can play a role in the heritable regulation of gene activity. Similar mechanisms are likely to play a role in the maintenance of a stable differentiated phenotype and might underlie results such as these.

On the other hand, dynamic regulatory factors must also be important, because the early heterokaryon experiments clearly suggest that any repression of gene activity can be overcome by diffusible factors. Perhaps the earliest and clearest identification of a factor that can play a role in the dynamic regulation of gene activity was the discovery of the helix–loop–helix transcription factor, MyoD. The presence of MyoD alone, introduced into a cell by transfection with appropriate expression vectors, is sufficient to activate muscle-specific genes from several distinct cell types. MyoD is also subject to positive autoregulation so that, once expressed in a cell, it tends to maintain its own expression, thus establishing a dynamic system for maintenance of the muscle-differentiated state.

Nevertheless, and not surprisingly, even this story is not so simple. In some cells, MyoD is not sufficient to activate muscle gene expression; other somatic cell hybrid experiments show that MyoD itself is subject to negative regulators specified by loci elsewhere in the genome. If these patterns of regulation also apply to other key regulatory genes, it would not be unexpected that the outcome of fusion experiments between distinct types of differentiated cells would depend on the parental cells, the interactions of structural modifications to chromatin and DNA, and the dynamic, diffusible regulatory factors pertinent to those cells.

HYBRIDS OF PLURIPOTENT CELLS

The behavior of hybrids of pluripotent cells has attracted considerable interest ever since lines of embryonal carcinoma (EC) cells were established *in vitro.* Indeed, the first description of established cultures of mouse EC cells included a report of the outcome of fusing EC cells and fibroblasts; in those experiments, the fibroblast phenotype predominated.

EC cells are the malignant, pluripotent stem cells of teratocarcinomas, which occur spontaneously in male mice of the 129 strain and in young human males, as testicular tumors of germ cell origin. Teratocarcinomas may contain an array of differentiated cell types corresponding to somatic cells of any of the three germ layers of the developing embryo, or to extraembryonic cell types of the yolk sac or the trophoblast (in man but not mice). The stem cell status of EC cells was first clearly demonstrated experimentally by the landmark study by Kleithsmith and Pierce (1964), and the relation of these cells to the inner cell mass or primitive ectoderm of the early stages of embryonic development, at least in the mouse, became evident

through a series of studies during the 1970s. Those studies laid the foundations for the derivation of embryonic stem (ES) cells directly from the blastocyst of both mouse and primate, including human, embryos. ES cells are evidently the "normal" counterparts of tumor-derived EC cells in both mice and humans. A closely related pluripotent cell type is the embryonic germ (EG) cell, lines of which have been derived from cultures of primordial germ cells from the genital ridges of both mouse and human embryos.

In the late 1970s, several groups reported experiments to investigate the consequences of fusing EC cells with somatic, differentiated cells. In those experiments, hybrids of EC cells with thymocytes retained an EC phenotype. Others found similar results in fusions of EC cells with various lymphoid cells. Typically, the hybrids extinguished differentiated markers of the lymphoid parents and retained pluripotency. In several cases in which the parental EC cells had lost their ability to differentiate (nullipotent EC cells), the hybrids regained that ability. The most likely explanation for this result is that the nullipotent EC cells had accumulated mutations that limited their ability to differentiate. Such mutations would be expected to provide EC cells with a strong selective advantage, because differentiation is typically accompanied by the loss of extended growth potential. Fusion with a wild-type cell could then allow genetic complementation and restore pluripotency, provided that the overall phenotype of the hybrid was that of an EC cell. Recently, a pluripotent human EC cell line, NTERA2, has been fused with a nullipotent line, 2102Ep. In that case, the hybrid cells did retain an ability to differentiate, suggesting that "loss-of-function" recessive mutations in 2102Ep were principally responsible for its inability to differentiate. However, although the hybrids differentiated, they did not give rise to neural differentiation, as the parental NTERA2 cells are well documented to do. Thus, the 2102Ep cells had evidently acquired dominant mutations that interfered with expression of that particular lineage in NTERA2 cells.

One circumstance in which the extinction of the somatic cell genes was variable was expression of the class 1, major histocompatibility complex (MHC) antigens. In mouse EC cells, the MHC antigens of the H2 complex are typically not expressed, although they are expressed in human EC cells. In some hybrids with lymphoid cells, expression of the somatic parent's H2 genes was suppressed; in others, it was not. In fusions of mouse EC cells with human lymphoid cells, most human chromosomes are lost, as generally occurs in mouse–human hybrids, and the hybrids retained an EC phenotype. However, in those retaining human chromosome 6, the human MHC antigens of the human lymphocyte antigen complex remained active.

Not all combinations of EC cells with somatic cells resulted in hybrids expressing an EC phenotype. Generally, hybrids with fibroblasts or fibroblastoid cells yielded fibroblastoid hybrids, as originally reported. In some hybrid combinations, for example, EC cell–hepatomas, both phenotypes were extinguished. However, intriguing results were obtained in EC cell–Friend erythroleukemia cell hybrids. Such hybrids generally expressed the phenotype of the erythroleukemia cells and were inducible for globin expression, which was specified by the EC-derived genome, as well as the erythroleukemia genome. Nevertheless, when the EC cell parent was tetraploid, some hybrids retained an EC phenotype. Apparently, an increased dosage of the EC genome was sufficient to overcome the dominant effect of the erythroleukemia cell.

REPROGRAMMING SOMATIC CELL NUCLEI WITH EC, ES, OR EG CELL CYTOPLASM

Not only in frogs, but also in mammals, nuclear transfer into enucleated eggs has resulted in the birth of live animals and illustrates the plasticity of nuclei derived from fully-differentiated somatic cells. The ability of the egg or oocyte to "reprogram" the somatic cell nucleus to such a profound extent is remarkable. These experiments raised the prospect of deriving embryonic stem cells genetically identical to a prospective patient, who might receive transplants of differentiated derivatives of those stem cells to replace diseased or damaged tissues. However, the technique is demanding and inaccessible for many researchers, and the availability of human donor eggs is limited and ethically problematic. An attractive alternative for both study and practical application would be to "reprogram" somatic cells using pluripotent ES, EG, or EC cells. However, despite the extensive earlier studies of EC–somatic cell hybrids, none clearly demonstrated reprogramming of the genome derived from the somatic cell parent. It is formally possible that the somatic genome was silenced, and that maintenance of the pluripotent state was actively dependent on continued gene expression from the EC cell-derived genome alone.

In more recent experiments, EG cells have been fused with thymocytes from transgenic mice carrying a neoR/lacZ transgene. As in the EC × thymocyte hybrids, EG–thymocyte hybrids expressing lacZ and drug resistance were isolated and retained a pluripotent EG-like phenotype. When the thymocytes came from transgenic mouse thymocytes engineered to express green fluorescent protein (GFP) under the transcriptional control of the Oct-4 promoter, the hybrids but not the thymocytes expressed GFP. Because Oct-4 is transcribed exclusively in germ cells, early embryos, and ES and EC cells, GFP should only be expressed when reprogramming of the thymocyte nucleus occurred. In addition, in both ES and EG hybrids with female thymocytes, and in similar EC hybrids, reactivation of the inactive thymocyte X-chromosome was observed. Thus, these experiments have now provided formal evidence that the somatic genome is indeed reprogrammed in these hybrids.

That the reprogramming ability is conserved between species was demonstrated in reciprocal fusions using mouse or human EC cells and cells of lymphocytic origin. Hybrids formed from fusion of the human T-cell line, CEM C7A, to mouse p19 EC cells expressed the endogenous human Oct-4 and Sox2 genes and adopted the morphology of the murine EC cells, with a high nuclear:cytoplasm ratio, prominent nucleoli, and growth in tight, well-defined adherent colonies. In the murine EC–human T-lymphocytes, there was considerable heterogeneity of expression between colonies and within a single colony over time. Thus, expression of human markers of cells of endoderm (collagen IV, laminin B1) and ectoderm (nestin) origin was noted in colonies that also expressed Oct-4 and Sox2. These data suggest that cross-species reprogramming had occurred in the hybrids and that subsequent spontaneous differentiation resulted in the human partner expressing the potential to adopt a fate not previously available to it. The reciprocal cross, using human 2102Ep EC cells and murine thymocytes, resulted in expression of the endogenous murine Oct-4 gene. Collectively, these data demonstrate that the ability to reprogram somatic nuclei is not the exclusive domain of the egg and oocyte, but can also be achieved by pluripotent ES, EG, and EC cells.

Interestingly, in a fusion of thymocytes with ES or EG cells, both EG and ES cells are capable of reprogramming thymocytes, but display different capacities for erasure of imprints. Imprinted genes are those for which the maternal and paternal alleles exhibit differential expression in somatic cells. Imprinting is established during gametogenesis, and most likely involves methylation at specific loci. After fertilization, the imprinted pattern of those specific genes is retained in all cells of the developing embryo except that, necessarily, such imprints are erased in the primordial germ cells (PGCs) and are reinstated prior to the completion of gametogenesis in preparation for the next generation. Thus, in ES cells derived from the blastocyst, monoallelic expression of imprinted genes is evident, but imprinting is absent from EG cells that have been derived from PGC after imprints have been erased. In EG–thymocyte hybrids, the H19 and p57Kip2 loci, normally methylated on the paternal allele and preferentially expressed from the maternal allele in thymocytes, were denuded of the paternal imprints. Similarly, the maternally methylated Peg1/Mest allele was demethylated in the EG–thymocyte hybrids, and both maternal and paternal alleles were expressed. Furthermore, ordinarily methylated but not imprinted genes, such as Aprt, Pgk2, and globin, were demethylated in these hybrids just as they are in PGCs. Thus, the EG cells appeared to possess the same capacity as PGC to erase imprints. By contrast, methylation at the imprinted loci, H19 and Igf2r, was maintained in ES–thymocyte hybrids. Similarly, it was also found that imprinting was not erased in EC–thymocyte hybrids, although the nonimprinted allele, normally silent in thymocytes, was expressed in the hybrids. These data strongly suggested that although extensive remodeling of the chromatin sufficient to establish pluripotency occurred, reprogramming does not extend to imprinting if the pluripotent partner itself retains imprinting. The erasure of imprints was also found to be dominant in ES–EG hybrids.

These observations on epigenetic imprinting in stem cell hybrids have relevance to the development of embryos derived after somatic nuclear transfer to enucleated oocytes, and fusions using ES, EG, and EC cells may serve as useful models of the process. The efficiency with which somatic nuclei generate living clones is notoriously low. Dolly the sheep, the first mammal cloned from a fully differentiated adult cell nucleus, was the single success in more than 300 nuclear transfer experiments. Epigenetic abnormalities that are the result of inappropriate imprinting are probably one important reason for the poor success rate and questionable health of clones living to term. A constellation of 70 to 80 genes was shown to be inappropriately expressed in mouse embryos cloned from adult nuclei. In addition, many cloned embryos are subject to developmental abnormalities indicative of faulty imprinting at several loci.

During normal embryogenesis, PGCs, which eventually differentiate into male or female gametes, are erased of imprinting. During the development of the gametes in embryos resulting from normal fertilization, correct imprinting is re-established. However, because a cloned individual does not result from fertilization by mature sperm of an egg, but rather from a donated diploid nucleus injected into an enucleate egg, the process of erasure and reinstatement of correct imprinting never occurs. The cell fusion experiments using ES, EG, and EC cells previously described may help to elucidate the process of imprinting and reprogramming, and to provide further information about why embryo cloning is a risky and inefficient process.

CELL FUSION AND THE DEMONSTRATION OF STEM CELL PLASTICITY

Recently, there have been myriad discussions and reports regarding adult stem cells and their apparent capacity not only to regenerate their own tissue of origin, but also to regenerate lineages other than those from which they derive. For example, hematopoietic stem cells and mesenchymal stem cells have been reported to generate neurons, muscle, hepatocytes, and a host of other tissues. Similarly, fetal neural cells have been reported to create hematopoietic and other tissues. A degree of controversy has surrounded these reports, and scientists have been at pains to design experiments that unequivocally demonstrate that a single such adult stem cell can generate cells of more than one lineage.

Cell fusion occurs spontaneously under appropriate conditions *in vivo*, as well as *in vitro*, and *in vivo* can be a normal physiological process during the development of several organs and tissues. For example, myoblast–myoblast and

myoblast–myotube fusions occur as part of normal skeletal muscle development, and fusion of trophoblast cells to form the giant cells occurs in the development of the placenta. Recent genetic studies in *Drosophila* and *Caenorhabditis elegans* have determined some of the genes required for these developmental fusion events.

The existence of genetically regulated, physiologically relevant cell fusion *in vivo*, as well as evidence of reprogramming of somatic cells following cell fusion, may confound many of the observations of adult stem cell plasticity. Two separate examples have recently been reported of spontaneous fusion of cells, in the absence of an external fusogenic agent, between populations of pluripotent stem cells and other cell types kept in co-cultures. For example, in mixed cultures using murine ES and genetically tagged bone marrow cells grown under conditions favoring the outgrowth of hybrids, it was revealed that ES–bone marrow hybrids could be isolated at a frequency of 10^{-5}–10^{-6}. Spontaneous fusion was dependent on the presence of IL-3 and the leukemia inhibitory factor, which obligates cytokines for hematopoietic lineages and the maintenance of pluripotency and self-renewal in ES cells, respectively. In similar experiments using mixed cultures of murine ES and genetically marked cells taken from fetal and adult mouse brain, it was also found that spontaneous hybrid formation occurred at ten-fold frequency. In both reports, resulting hybrids displayed the morphology and pluripotency of the ES partner. Previously the somatic partner in these hybrids was restricted in its developmental fate to producing the repertoire of hematopoietic lineages or central nervous system lineages, respectively; however, fusion with the ES cell allowed expression of new potential fates. These spontaneously formed hybrid cells were capable of contributing to all three germ layers after injection into blastocysts. Thus, in any demonstration of lineage plasticity of adult stem cells, it becomes important to rule out the possibility of fusion with another endogenous pluripotent stem cell that reprograms the test cell.

A further development in cell fusion has recently been documented in two reports examining the nature of hepatocytes derived from hematopoietic stem cells, previously one of the most robust arguments for adult stem cell plasticity. Using mice engineered to be susceptible to liver degeneration through tyrosinemia because of ablation of the fumarylacetoacetate hydrolase gene, the regeneration of hepatocytes from transplanted, lineage-depleted bone marrow was unequivocally shown. However, ordinarily, the liver contains a subset of polyploid cells, suggesting that cell fusion may be a characteristic feature of this organ. The two follow-up studies report that the hepatocyte outgrowth as a result of hematopoietic stem cell transplantation almost certainly occurred because of the fusion of hematopoietic cells with pre-existing hepatocytes. In another study, some of the regenerated liver cells were diploid rather than tetraploid, as would be expected when two diploid cells fuse. This suggests that it is possible to generate a diploid cell from a tetraploid hybrid and still maintain the reprogrammed phenotype.

The identity of the fusion partner derived from bone marrow was not determined in these reports. Nevertheless, the authors speculate that it is probably not the hematopoietic stem cell itself but a later progenitor. In the liver regeneration model, the authors speculate that the hematopoietic partner cells are phagocytic Kupffer cells, macrophages, B- or T-cells; this view is supported by the finding that liver regeneration occurred after the hematopoietic lineages had been repopulated. However, because liver injury did not mobilize a similar response, it is unlikely that this is a generalized response to tissue damage; rather, it may be the result of the artificial constraints of the experimental design.

Others have observed indications of engraftment in distant tissues by cells introduced during the transplantation of donated organs or tissues. For example, Y-chromosome-positive cells with apparent neuronal or cardiomyocyte function have been noted in the brains and hearts, respectively, of female bone marrow transplant recipients. These observations have been interpreted as evidence of transdifferentiation of the incoming donor cells, and subsequent population of host organs. However, given the findings described previously, it now seems plausible that these may have been the result of spontaneous fusion of host and donor cells.

SUMMARY

The study and use of cell fusion has an extensive history. It has been an invaluable tool in somatic cell genetics, and it has highlighted the complexity of the mechanisms that regulate the maintenance of the determined and differentiated states. Although it is difficult to make strong claims that the study of cell hybrids has contributed substantially to understanding those mechanisms, the ability of pluripotent stem cells to reprogram somatic nuclei to a primitive, pluripotent state increases the potential of achieving somatic cell reprogramming in an efficient manner for therapeutic purposes without resorting to nuclear transfer to oocytes—so-called therapeutic cloning. The phenomenon of spontaneous fusion is also an issue that now has to be addressed in the analysis of any claims for plasticity of otherwise lineage-restricted, adult stem cells.

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KEY WORDS

Cell fusion The process whereby the cell membrane between two juxtaposed cells breaks down and reforms

to incorporate the cytoplasm and nuclei of both cells into a single, viable cell.

Heterokaryon The immediate result of fusing two different cells which yields a single cell with two or more identifiable intact nuclei, one from each parental cell.

Hybrid cell The cell that arises when a heterokaryon resulting from cell fusion enters mitosis, with breakdown of the nuclear membranes of the individual nuclei and formation of a single nucleus in each daughter cell containing the genetic material from the original parental cells. Note, however, that there is often significant genome loss during this and subsequent divisions, so the hybrid cell may not contain all of the genetic material from each parental cell.

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Chapter 12

How Cells Change Their Phenotype

David Tosh and Marko E. Horb

INTRODUCTION

Until recently, it was thought that once a cell had acquired a stable differentiated state, it could not change its phenotype. We now know this is not the case, and over the past few years a plethora of well-documented examples have been presented whereby already differentiated cells or tissue-specific stem cells have been shown to alter their phenotype to express functional characteristics of a different tissue. In this chapter, we examine evidence for these examples, comment on the underlying cellular and molecular mechanisms, and speculate about possible directions of research.

METAPLASIA AND TRANSDIFFERENTIATION

Definitions and Theoretical Implications

"Metaplasia" is defined as the conversion of one cell type to another, and it can include conversions between tissue-specific stem cells. "Transdifferentiation," on the other hand, refers to the conversion of one differentiated cell type to another, and should therefore be considered a subset of metaplasia. Historically, metaplasia has been the term used by pathologists, but in recent year's transdifferentiation has become the favored term, even when discussing the conversion of tissue-specific stem cells to unexpected lineages. Within the medical community, the idea of metaplasia is not controversial, but in the scientific community, some skepticism still surrounds the phenomenon of transdifferentiation—it being attributed to tissue culture artifacts or cell fusion. Nevertheless, it is important to study metaplasia and transdifferentiation to gain a better understanding about the regulation of cellular differentiation, which may lead to new therapies for a variety of diseases, including cancer.

WHY STUDY TRANSDIFFERENTIATION?

Regardless of which definition is applied, we consider the study of transdifferentiation and metaplasia to be important for four reasons. First, it allows us to understand the normal developmental biology of the tissues that interconvert. Most transdifferentiations occur in tissues that arise from neighboring regions in the developing embryo, and are therefore likely to differ in the expression of only one or two transcription factors. If the genes involved in transdifferentiation can be identified, then this might shed some light on the developmental differences that exist among adjacent regions of the embryo.

Second, it is important to study metaplasia because it predisposes to certain pathological conditions, such as Barrett's metaplasia (see later sections for further details). In this condition, the lower end of the esophagus contains cells characteristic of the intestine, and there is a strong predisposition to adenocarcinoma. Therefore, understanding the molecular signals in the development of Barrett's metaplasia will help to identify the key steps in neoplasia and may provide us with potential therapeutic targets, as well as diagnostic tools.

Third, understanding transdifferentiation will help to identify the master switch genes and thus allow us to reprogram stem cells or differentiated cells for therapeutic purposes. The fourth reason is that we will be able to identify the molecular signals for inducing regeneration, and therefore promote regeneration in tissues that otherwise do not regenerate (e.g., limb regeneration).

EXAMPLES OF THE PHENOMENON

Despite the controversy surrounding transdifferentiation, there are numerous examples that exist in both humans and animals; here we will focus on a select few. The examples we have chosen to examine in detail include the conversions of pancreas to liver, liver to pancreas, esophagus to intestine, iris to lens, and bone marrow to other cell types. Other

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chapters in this book also describe some of these, as well as additional examples, in more detail.

Pancreas to Liver

The conversion of pancreas to liver is one well-documented example of transdifferentiation. This type of conversion is not surprising, since both organs arise from the same region of the endoderm and both are thought to arise from bipotential cells in the foregut endoderm. In addition, the organs share many transcription factors, displaying their close developmental relationship. The appearance of hepatocytes in the pancreas can be induced by different protocols, including feeding rats a copper-deficient diet with the copper chelator Trien, overexpressing keratinocyte growth factor in the islets of the pancreas, or feeding animals a methionine-deficient diet and exposing them to a carcinogen. It has also been observed naturally in a primate, the vervet monkey. Although the functional nature of the hepatocytes has been examined in detail, until recently the molecular and cellular basis of the switch from pancreas to liver was poorly understood.

Two *in vitro* models have been produced for the transdifferentiation of pancreas to liver. The first model uses the pancreatic cell line AR42J, and the second uses mouse embryonic pancreas tissue in culture; both rely on the addition of glucocorticoid to induce transdifferentiation. AR42J cells are amphicrine cells derived from azaserine-treated rats, and they express both exocrine and neuroendocrine properties. That is, they are able to synthesize digestive enzymes and express neurofilament. The dual nature of this cell line is evident in that, when exposed to glucocorticoid, they initially enhance the exocrine phenotype by producing more amylase, but when cultured with hepatocyte growth factor and activin A, the cells convert to insulin-secreting β-cells. These properties of the AR42J cells suggest that they are an endodermal progenitor cell type with the potential to become exocrine or endocrine cell types.

The transdifferentiated hepatocytes formed from pancreatic AR42J cells express many of the proteins normally found in adult liver—for example, albumin, transferrin, and transthyretin. They also function as normal hepatocytes; in particular, they are able to respond to xenobiotics (e.g., they increase their catalase content after treatment with the peroxisomal proliferator, ciprofibrate). Although the mouse embryonic pancreas also expresses liver proteins after culture with dexamethasone, it is not clear whether the same cellular and molecular mechanisms are in operation as in the AR42J cells. It is possible that, rather than the hepatocytes arising from already differentiated cell types, the liver-like cells are derived from a subpopulation of pancreatic stem cells.

To determine the cell lineage of hepatocyte formation from pancreatic AR42J cells, we performed a lineage experiment based on the perdurance of green fluorescent protein (GFP) and used the pancreatic elastase promoter. After the transdifferentiation, some cells that expressed GFP also contained liver proteins (e.g., glucose-6-phosphatase). This result suggests that the nascent hepatocytes must have once had an active elastase promoter; therefore, they were differentiated exocrine cells. To elucidate the molecular basis of the switch in cell phenotype, we determined the expression of several liver-enriched transcription factors. Following treatment with dexamethasone, C/EBPβ became induced, the expression of the exocrine enzyme amylase was lost, and liver genes (e.g., glucose-6-phosphatase) were induced. These properties of C/EBPβ make it a good candidate to be an essential factor involved in the transdifferentiation of pancreas to liver. Indeed, C/EBPβ is sufficient to transdifferentiate AR42J cells to hepatocytes. Therefore, C/EBPβ appears to be a good candidate for the master switch gene distinguishing liver and pancreas.

Liver to Pancreas

The numerous examples of pancreas-to-liver transdifferentiation suggest that the reverse switch should also occur readily; nevertheless, examples of this type of conversion are infrequent. The presence of pancreatic tissue in an abnormal location is known as heterotopic, accessory, or aberrant pancreas, and the frequency has been reported to range from 0.6 to 5.6%. In most cases (70–90%), the heterotopic pancreas is found in the stomach or intestine and is considered to be an embryological anomaly. In contrast, intrahepatic pancreatic heterotopia has only been reported in six individuals, comprising less than 0.5% of all cases of heterotopic pancreas. In general, heterotopic pancreatic tissue can be composed of exocrine, endocrine, or both types of cells. In almost every case of pancreatic heterotopia in the liver, however, only exocrine cells are present; only one case describes the presence of endocrine cells. Unlike the other cases of accessory pancreas, these rare incidents of intrahepatic pancreatic tissue cannot be explained as the result of a developmental error. In fact, in most of these cases, the patients were diagnosed with cirrhosis, suggesting that the heterotopic pancreas arose as a metaplastic process. Results with the animal models concur.

In other animals, pancreatic exocrine tissue can be induced in the liver by feeding rats polychlorinated biphenyls or by exposing trout to various carcinogens, such as diethylnitrosamine, aflatoxin B_1, or cyclopropenoid fatty acid. In these examples, the hepatic exocrine tissue is most often associated with tumors or injury, such as hepatocellular carcinomas (which arise from hepatocytes) or cholangiolar neoplasms (which arise from the bile duct), or adenofibrosis. Much like the human cases, these results suggest that, during carcinogenesis, a metaplastic event occurs that generates pancreatic tissue. Indeed, pancreatic metaplasia in trout can be inhibited by the addition of the glucosinolate indole-3-carbinol, a known anticancer agent. Whether

inhibiting metaplasia prevents neoplasia remains to be determined. The ability of one cell (liver) to transdifferentiate into another (pancreas), no matter how rare, suggests that it should be possible to identify the molecular signals involved in switching a cell's phenotype, and thus to learn how to control and direct this conversion for therapeutic purposes.

Two recent reports have shown that it is possible to convert liver cells experimentally into pancreatic cells. Each has used a different approach to bring about transdifferentiation—by changing either the extracellular or the intracellular environment. In the first example, hepatic oval cells were isolated and maintained in tissue culture media supplemented with leukemia inhibitory factor (LIF). Upon the removal of LIF and the addition of high concentrations of glucose (23 mM) in the medium, the oval cells transdifferentiated to pancreatic cells. The oval cells were converted into a variety of pancreatic cell types, including glucagon, insulin, and pancreatic polypeptide-expressing cells. Functionally, these oval cell-derived endocrine cells were able to reverse hyperglycemia in streptozotocin-induced diabetes. The mechanism whereby glucose induces the transdifferentiation is not known, though previously it was shown that glucose can promote the growth and differentiation of β-cells in the normal pancreas; perhaps a similar mechanism operates here.

In the second example, hepatic cells (either *in vivo* or *in vitro*) were induced to transdifferentiate by overexpression of a superactive form of a known pancreatic transcription factor, Pdx1. Pdx1 is expressed early in the endoderm prior to overt morphological development of the pancreas, and has been shown to play a fundamental role in the development of the entire pancreas. Although a previous study showed that continuous overexpression of the unmodified Pdx1 in the liver increased hepatic insulin production, it is not known if this represents a true transdifferentiation or simply the activation of the insulin gene.

In the study using modified Pdx1, the transdifferentiation appears to be relatively complete in that both exocrine cells and endocrine cells were produced, including insulin, glucagon, and amylase-expressing cells. On its own, Pdx1 is unable to convert liver to pancreas and requires an extra activation domain, VP16. This might be because of the lack of appropriate protein partners or the presence of some inhibitory proteins in the liver cells, since sequence-specific transcription factors are known to require other tissue-specific coactivators to stimulate transcription. To overcome this problem, the VP16 activation domain was fused to Pdx1—the VP16 activation being able to activate transcription directly by binding to various coactivators, as well as the basal transcription machinery, eliminating the need for other tissue-specific proteins. When Pdx1–VP16 was overexpressed in the liver, using the liver-specific promoter transthyretin, it was able to induce the transdifferentiation of liver to pancreas. This is an example whereby a known transcription factor essential for pancreas development was engineered to act as a master switch gene.

In conclusion, the preceding results demonstrate that it is possible to transdifferentiate liver cells, whether fully differentiated or not, into pancreatic cells. Since the liver has the ability to regenerate, this tissue can provide an abundant resource for the production of pancreatic cells with the aim of curing diabetes. The first study shows that an extracellular factor, glucose, can be used; the second reveals that a modified intracellular tissue-specific transcription factor, Pdx1–VP16, is sufficient. These studies suggest that if we can identify the key factors involved in the physiological regulation of the adult pancreas, as well as in embryonic development, this will play an important role in understanding and promoting the transdifferentiation of various cell types into pancreas. However, overexpression of a single transcription factor may not be sufficient; the ability to change a cell's phenotype may therefore require the use of modified or engineered transcription factors (e.g., Pdx1–VP16) that can artificially recruit the transcriptional machinery.

Barrett's Metaplasia

Barrett's metaplasia (or Barrett's esophagus, as it is sometimes called) is the clinical situation in which intestinal cells are found in the tissue of the lower end of the esophagus. In the strictest terms, it is the conversion of stratified squamous epithelium to columnar epithelium, and is characterized by the presence in biopsy material of acid mucin-containing goblet cells. The importance of Barrett's metaplasia stems from the rise in apparent incidence of the disease, and its risk associated with the development of adenocarcinoma of the esophagus. It is not known why metaplastic cells have a greater disposition toward neoplastic progression.

One of the proposed mechanisms for development of Barrett's metaplasia is gastroesophageal reflux. It is assumed that prolonged acid reflux from the stomach (generally with bile acids) promotes damage to the epithelia at the end of the esophagus. Presumably, in the early stages of the disease, the normal stratified squamous epithelium is replaced. Eventually, re-epithelialization results in the formation of columnar, as opposed to stratified, squamous epithelium, most likely because of repeated exposure to an acid environment. It is not clear whether the different intestinal cell types formed in the esophagus arise from the same stem cell in the basal layer, or whether there is a transdifferentiation of columnar cells to goblet cells. It is also not understood why some patients with efflux disease do not go on to develop Barrett's metaplasia.

The molecular events underlying Barrett's metaplasia are not well-understood. However, good candidate genes include the *caudal*-related homeobox genes *cdx1* and *cdx2*. Several lines of evidence support this statement. First, both

genes are expressed in the intestine (but not in the stomach or in the esophagus) and are known to be important in regulating intestine-specific gene expression. Second, colonic epithelium is transformed to squamous epithelium (similar to the esophagus) in mice haploinsufficient for *cdx2*. Third, and more recently, it was found that ectopic expression of *cdx2* in the stomach can induce intestinal metaplasia, and there is some evidence for early expression of *cdx2* in patients with Barrett's metaplasia. These results suggest that *cdx* genes may provide a target for therapeutic intervention in Barrett's metaplasia.

Regeneration

The idea of regenerative medicine or tissue engineering mainly implies using tissue-specific stem cells to replace damaged or "lost" organs. However, as more examples of the plasticity of differentiated cells become known, it may be more feasible to use them than to use stem cells. Historically, the classical example of transdifferentiation occurs during lens regeneration in newts, where the dorsal iris-pigmented epithelium (IPE) is converted to lens. In other species, it may be a different source of cells that undergoes transdifferentiation to lens, such as the outer cornea in *Xenopus laevis.* In a similar fashion, regeneration of the neural retina occurs by transdifferentiation of the retinal-pigmented epithelium (RPE) in various vertebrates. In both cases, the regeneration has been shown to occur in adult newts, as well as in other vertebrate embryos, including chick, fish, and rat.

On removal of the lens (lentectomy) only the dorsal IPE transdifferentiates into lens, in three phases—dedifferentiation, proliferation, and transdifferentiation—even though both the dorsal and ventral IPE have the potential to become lens. It has been shown that the dorsal, and not the ventral, IPE expresses Pax6, Prox1, and FGFR-1, suggesting that these factors may play an important role in inducing the transdifferentiation. Indeed, inhibition of FGFR-1 will block the transdifferentiation of IPE to lens, and in *Xenopus*, it has been shown that FGF-1 can induce the transdifferentiation of outer cornea to lens. In agreement with this, retinal regeneration in chick embryos can be promoted by the addition of either FGF-1 or FGF-2, but not other growth factors such as TGFβ. These results show that the microenvironment in which a cell resides plays a critical role in regulating transdifferentiation.

Why one cell changes its phenotype (dorsal IPE), but another does not (ventral IPE), in response to exogenous growth factors may depend on the competence of each cell (e.g., the presence of the appropriate receptors). It is possible that FGFs may be responsible for inducing dedifferentiation of the tissue, and that the expression of particular transcription factors induces both proliferation and transdifferentiation. This classical model of lens regeneration illustrates that the molecular signals involved in transdifferentiation can be identified and used to promote regeneration of cells normally considered unable to alter their phenotype.

Bone Marrow to Other Cell Types

Some cell-type conversions using bone marrow-derived stem cells have been shown to occur across what was previously considered to be germ-line boundaries (i.e., mesoderm to endoderm). In this situation, it is not evident whether the cell must first become a different stem cell and then differentiate along a different pathway or if it directly transdifferentiates to another phenotype. However, some doubt has been cast on these observations, and it has been suggested that the result is caused by an artifact from fusion of the circulating hematopoietic stem cells with resident cells.

DEDIFFERENTIATION AS A PREREQUISITE FOR TRANSDIFFERENTIATION

A question arises: if transdifferentiation is to occur, must the parent cell lose its phenotype before acquiring a new identity? In some examples (IPE to lens), there is an intermediate phenotype in which the cells do not express markers for either cell type (Figure 12-1B). However, examples

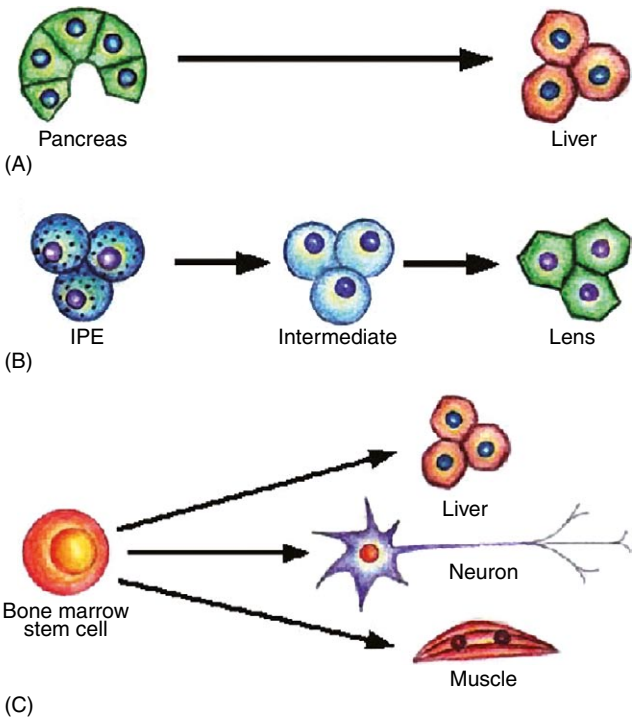


FIGURE 12-1 Examples of transdifferentiation. Transdifferentiation can occur in different stages. (A) Transdifferentiation of pancreas to liver can occur without cell division or an intermediate phenotype. (B) Transdifferentiation of pigment epithelium to lens requires an intermediate stage in which the cell does not possess the characteristics of either phenotype. (C) The pluripotency of stem cells is shown by their ability to convert to different cell lineages. In this example, the switch is direct and there is no conversion to another tissue-specific stem cell.

of direct transdifferentiation do occur. Perhaps the best example is the transdifferentiation of pancreas to liver (pancreatic exocrine to hepatocyte) (Figure 12-1A). Whether a cell undergoes transdifferentiation directly, through a dedifferentiated state, or through a stem cell, may vary depending on which cell types are being studied (Figure 12-1). In other words, does the parent cell contain the necessary information to change its phenotype directly, or does it require the synthesis of new proteins? In direct transdifferentiation, the cell's competency is already established, and it is the removal of an inhibitor or the addition of an activator that pushes the fate of the cell over the final hurdle. For dedifferentiation and stem cell intermediates, it may be necessary to establish the competency of the parent cell before it can undergo transdifferentiation. Further studies examining the transdifferentiation potential of individual transcription factors and various cell types will help to bring about an understanding of the rules of transdifferentiation.

HOW TO CHANGE A CELL'S PHENOTYPE EXPERIMENTALLY

The ability to change a cell's phenotype will greatly facilitate the design of therapies for diseases such as diabetes, liver failure, and neurodegenerative disorders (e.g., Parkinson's disease). We suggest six steps to follow to try and change a cell's phenotype experimentally.

1. Identify Potential Factors to Induce Transdifferentiation

Transdifferentiation may be achieved in several ways using extracellular growth factors, individual transcription factors, or combinations of the two. An understanding of how individual organs or cell types form will help to identify those molecular factors that can be used to direct the transdifferentiation of other cell types. We believe that those factors essential for the initial development of an organ will work best, since they sit at the top of the hierarchy of the signaling cascade. Functional screens, such as those used previously to identify novel mesoderm-inducing factors, should aid the identification of new factors with the potential to direct transdifferentiation.

2. Choose a Cell Type to Convert

In many cases of transdifferentiation, only certain cells can undergo a specific transdifferentiation, suggesting that there are restrictions on whether a cell has the competence to undergo transdifferentiation. Therefore, choosing the cell type initially is important. We suggest that using closely-related cell types will greatly improve the chances for transdifferentiation. Examples include the use of pancreatic AR42J cells in the conversion of pancreas to liver. Ultimately, either primary cultures of well-defined cell types or *in vivo* experiments will be necessary for this to be of therapeutic use.

3. Choose the Method of Overexpression

It is important to determine whether continuous or limited overexpression of a particular factor is required. On the one hand, tissue-specific promoters will allow the factor to be expressed for only a relatively short time; upon transdifferentiation, the promoter will no longer be active. Ubiquitous promoters, on the other hand, will express the chosen factor continuously and may produce undesired results. Many transcriptional regulators are expressed only transiently, and require a strict temporal regulation for proper development to occur. For example, continuous overexpression of *Hlxb9* in the normal pancreas interferes with the differentiation of both exocrine and endocrine cells. Thus, the use of the constitutive promoters, such as cytomegalovirus, may not be suitable. We believe that the use of tissue-specific promoters is best suited to the type of experiments described here, to prevent the chosen factor from interfering with the proper differentiation of the new phenotype.

4. Identify Whether a Modification of the Factor is Required

It is possible that after identifying the factor, no transdifferentiation occurs. There may be several reasons for this, but we suggest testing a superactive version of the same factor before dismissing it. The easiest way to do this is to make use of a strong activation domain that has already been characterized, such as VP16. Whether it is fused to the N- or C-terminus should not matter, but we suggest that the VP16 be fused to the whole open reading frame of the transcription factor, and not just to the DNA-binding domain.

5. Characterize the New Phenotype

Initially, use of a reporter construct will greatly help the identification of a successful conversion—for example, the use of the elastase promoter driving GFP in the conversion of liver to pancreas. It is best to initially use a promoter expressed in all cell types in a particular organ rather than one specific to an individual cell type within that organ. Three questions can be addressed under this heading. First, is the transdifferentiation specific to producing a single cell type or, if the organ contains numerous cell types, are several types produced? In the case of the transdifferentiation of liver to pancreas, ectopic expression of Pdx1–VP16 produces more than one type of pancreatic cell. Second, what

is the identity of the new cell type, and can the loss of the other phenotype be demonstrated? Third, is the transdifferentiation stable? For a cell-type conversion to be a true transdifferentiation, the phenotype of the new cell must be stable.

6. Test the Transdifferentiation Activity in Other Cell Types

As mentioned previously, in many instances only a subset of cells can undergo a particular transdifferentiation. Therefore, it is essential to identify which cells can be transdifferentiated, and why one cell is able to respond but another is not. An understanding of the competence of individual cells will lead to a greater understanding of what is necessary for transdifferentiation to occur—for example, is dedifferentiation a necessary prerequisite? The question of whether it will be possible to direct the transdifferentiation of cells into one particular cell type, such as an insulin-secreting β-cell, remains to be seen.

SUMMARY

The recent demonstration that adult stem cells, and even differentiated cells, are more versatile than previously thought means that abundant tissue for therapeutic purposes can be obtained from these cell types. Since some scientists and members of the public are averse to the use of embryonic stem cells for any form of clinical treatment, using either adult stem cells or differentiated cells induced to transdifferentiate may obviate the use of embryonic tissue. In the end, the ethical issues raised by some may not apply to transdifferentiated cells.

ACKNOWLEDGMENTS

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KEY WORDS

Master switch gene A transcription factor that is sufficient to induce the transdifferentiation of one cell type to another; is associated with the loss of one set of differentiated properties (e.g., pancreatic) and the gain of another phenotype (e.g., hepatic). Master switch genes are normally involved in coordinating the differentiation of a particular cell fate through the activation of various downstream targets.

Metaplasia The conversion of one cell type to another; can include conversions between tissue-specific stem cells.

Transdetermination The all-or-none conversion of one regenerating body structure into another.

Transdifferentiation The conversion of one differentiated cell type to another; a subset of metaplasia.

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Part III

Tissue and Organ Development

Chapter 13

Differentiation in Early Development

Susana M. Chuva de Sousa Lopes and Christine L. Mummery

INTRODUCTION

During the first cleavage divisions, totipotent blastomeres of the mammalian embryo segregate and eventually become committed to the extraembryonic, somatic, and germline lineages, losing developmental potency. In mice and humans, pluripotent embryonic cells can be isolated from early embryos and maintained in culture as embryonic stem (ES) cells. In this chapter, we review the development of the mammalian embryo during pre-implantation and the earliest post-implantation stages as a basis for understanding the developmental potency of ES cells, although it is important to realize that, despite nearly 40 years of research, a number of basic questions on early mammalian development still remain unanswered. On the one hand, this is due to the small size of early mammalian embryos and the effort that is required to obtain them in the large numbers necessary, until recently, for analysis of gene and protein expression. On the other hand, it is because implantation (and later placentation), through which the embryo establishes (and maintains) a physical connection with the mother, makes embryos relatively inaccessible; it has not been possible to mimic implantation *in vitro* or monitor these developmental stages *in vivo*. However, advances in techniques for analysis of gene expression in small sample sizes, *in vitro* fertilization, clonal analysis of cultured embryos, and the use of genetic markers are providing new clues on key developmental events and allowing important questions to be addressed.

PRE-IMPLANTATION DEVELOPMENT

In mammals, fertilization occurs in the oviduct where sperm encounters and fuses with the oocyte. As a result, the oocyte nucleus, which had been arrested in metaphase II, completes meiosis, and the two parental pronuclei fuse to form the diploid zygotic nucleus (Figure 13-1A). Progressive DNA demethylation of both paternal and maternal genomes (excluding the genomic imprints) begins after fertilization, leading to epigenetic reprogramming. Transcription of the embryonic genome starts at the two-cell stage in mice and at the four- to eight-cell stage in humans. Until then the embryo relies solely on maternal mRNA, but after activation of the embryonic genome, maternal transcripts are rapidly degraded, although maternally-encoded proteins may still be present and functionally important. The embryo continues cleaving without visible growth (Figure 13-1).

Cell Polarization Occurs During Compaction

At the eight-cell stage in mice and the eight- to 16-cell stage in humans, the embryo undergoes a process known as compaction to become a morula, a compact smooth spherical structure (Figure 13-1D). All blastomeres flatten, maximize their contacts, and become polarized. Their cytoplasm forms two distinct zones: the apical domain accumulates endosomes, microtubules, and microfilaments, whereas the nucleus moves to the basal domain. Furthermore, gap junctions form basally ensuring communication between blastomeres, and numerous microvilli and tight junctions are formed apically.

The next cleavage plane of some blastomeres is perpendicular to their axis of polarity, resulting in two cells with different phenotypes. One daughter cell is located inside the embryo, is small and apolar, and contains only basolateral elements. The other daughter cell is located at the surface of the embryo, is larger and polar, and contains the entire apical domain of the progenitor cell and some basolateral elements. These polar cells inherit the region containing the tight junctions, thereby creating a physical barrier between the inner apolar cells and the maternal environment.

Blastocyst Formation (Cavitation)

After compaction, the presumptive trophectoderm (TE) cells form the outer layer of the embryo. Intercellular

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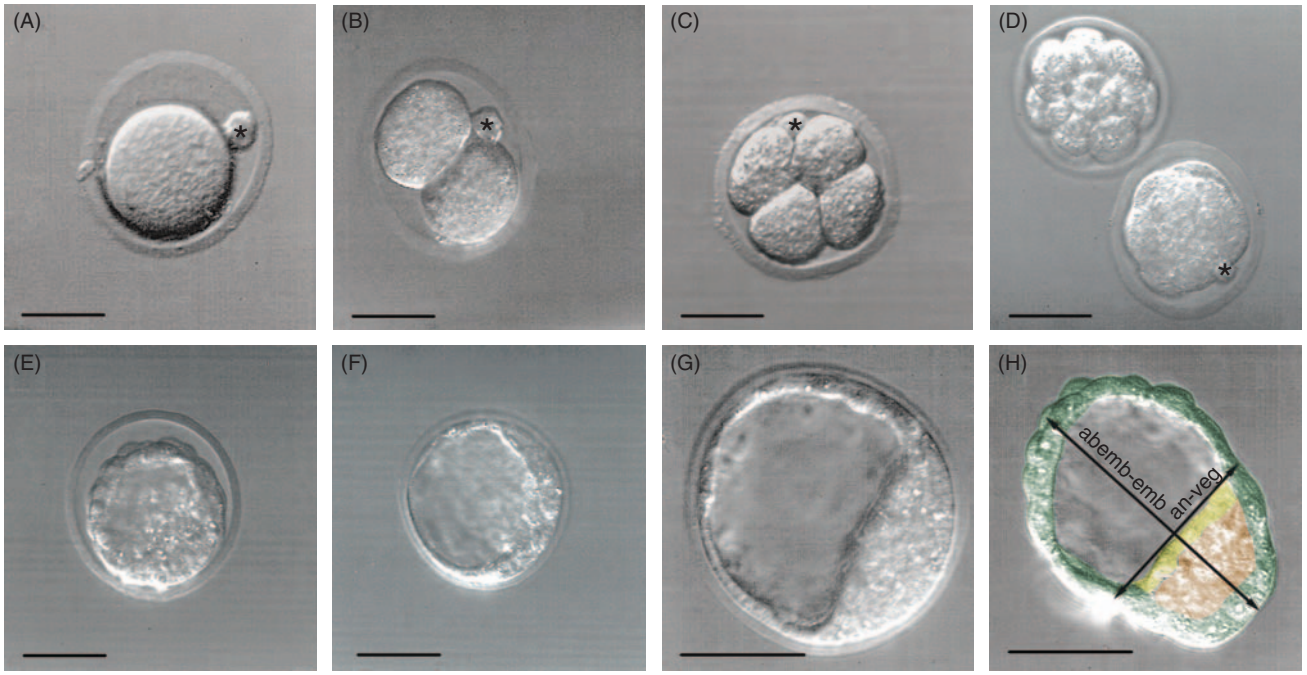


FIGURE 13-1 Mouse preimplantation development. After fertilization, the two parental pronuclei fuse to form the zygote (A). The embryo cleaves, forming a two-cell (B), four-cell (C), and eight-cell embryo. The embryo then undergoes compaction to become a smooth spherical structure, the morula (D). Note that the second polar body remains attached to the embryo (*). The blastocoelic cavity then develops on one side of the embryo to form an early blastocyst (E). The cavity enlarges, occupying most of the expanded blastocyst (F, G). Around embryonic day E4.5, the late blastocyst reaches the uterus, "hatches" from the zona pellucida, and is ready to implant (H). The late blastocyst consists of three cell sub-populations: the trophectoderm (green); the inner cell mass (orange); and the primitive endoderm (yellow). In the blastocyst three axes can be defined: the embryonic-abembryonic (abemb-emb); the animal-vegetal (an-veg); and a third axis on the same plane but perpendicular to the an-veg axis. Photomicrographs courtesy of B. Roelen.

contacts strengthen between these cells, and a true epithelium is formed. This thin single-cell layer develops a continuum of junctional complexes, including gap junctions, desmosomes, and tight junctions. Furthermore, the composition of the basal and apical membranes becomes more distinct, with Na^+/K^+-ATPases accumulating in the basal membrane. These ion pumps actively transport sodium ions into the embryo, which leads to accumulation of water molecules. A fluid-filled cavity, the blastocoelic cavity, is thus created on one side of the embryo in a process known as cavitation (Figure 13-1E). The presumptive inner cell mass (ICM) cells stay closely associated during this process, not only because of gap junctions, tight junctions, and interdigitating microvilli between the cells, but also because processes from TE cells fix the ICM to one pole of the embryo and partially isolate it from the blastocoel. The intercellular permeability seal of the TE cells prevents fluid loss, and as a consequence the blastocoelic cavity gradually expands to occupy most of the blastocyst between the 64- and 128-cell stage (Figure 13-1E–G). At this stage, the embryo is not radially symmetric around the embryonic-abembryonic axis, but is bilaterally symmetric (the zona pellucida is slightly oval).

The outer TE layer and the ICM are composed of descendants of the outer and inner cell population of the morula, respectively. The TE in turn consists of two subpopulations: the polar TE contacts the ICM and the mural TE surrounds the blastocoelic cavity. The TE descendants give rise to extraembryonic structures, such as the placenta, but do not contribute to the embryo proper. The ICM also consists of two cell sub-populations mixed in a salt and pepper fashion. However, due to adherence differences, one of the sub-populations (GATA6-positive cells) segregates to the surface of the ICM where it contacts the blastocoelic cavity and differentiates to primitive endoderm, which is also an extraembryonic tissue. The other cell population gives rise to the embryo proper, as well as the extraembryonic mesoderm that contributes to form the visceral yolk sac, amnion, and the allantois, a structure that will form the umbilical cord. An overview of cell lineage relationships in the early mouse is shown in Figure 13-2. During preimplantation development (three to four days in mice, five to seven days in humans), the embryo has traveled through the oviduct inside the zona pellucida, a protective glycoprotein coat. Reaching the uterus, the blastocyst "hatches" from the zona pellucida and is ready to implant (Figure 13-1H).

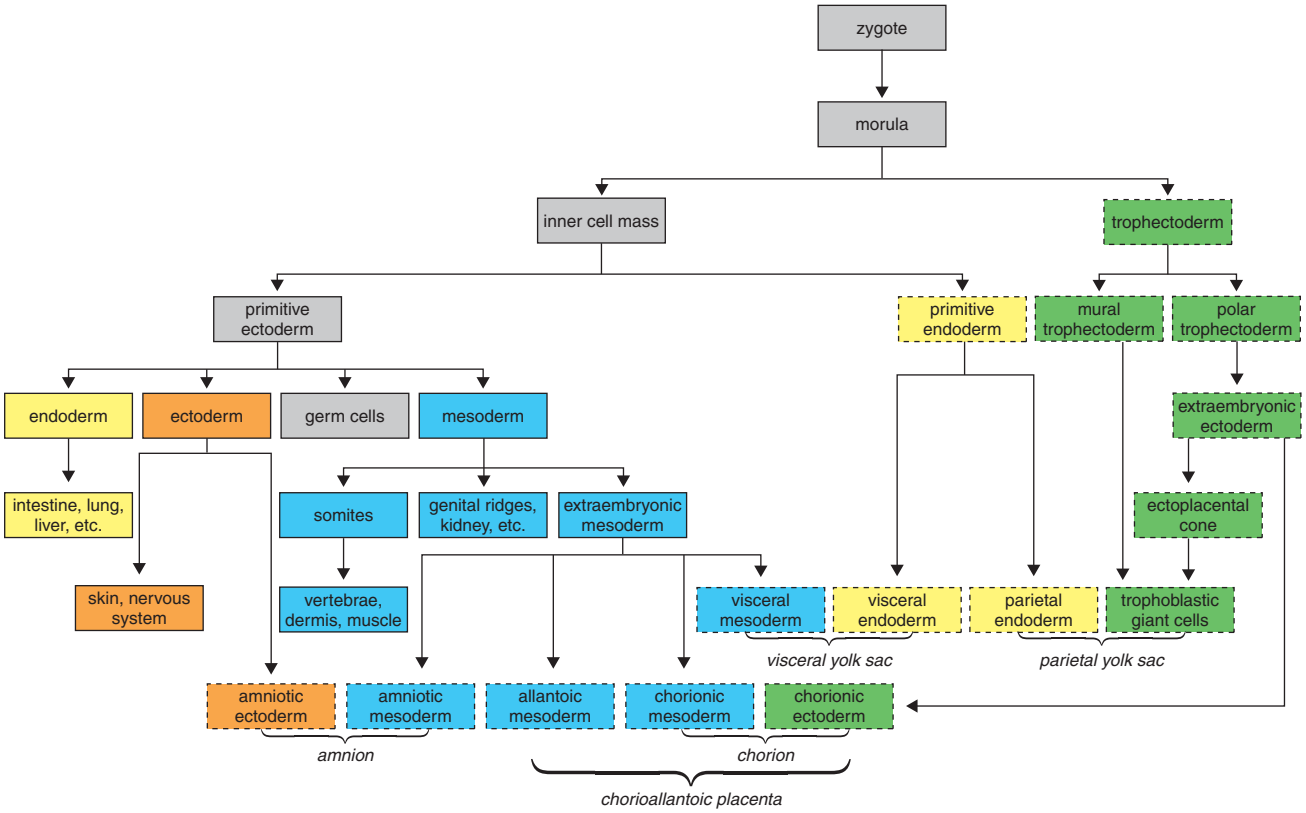


FIGURE 13-2 Cell lineages in mouse development. Trophectoderm-derived tissues are depicted in green, endoderm-derived tissues in yellow, ectoderm-derived tissues in orange, and mesoderm-derived in blue. The cell/tissues represented in gray are regarded as pluripotent. All extraembryonic tissues are enclosed by hatched lines, whereas embryonic tissues are enclosed by solid lines. Adapted from Nagy *et al.*, 2003.

Stages of mouse and human pre-implantation development are summarized in Table 13-1.

Axis Specification During Pre-implantation in the Mouse?

In lower vertebrates, the body axes are already specified (determined) in the undivided egg or very soon thereafter, whereas in mammalian embryos, axis specification is thought to be completed only during gastrulation. This view is supported by the observation that the mammalian embryo is extremely plastic, ignoring disturbances such as the removal or reaggregation of blastomeres. The prevailing concept, therefore, became one of no embryonic pre-patterning before gastrulation. However, several studies have challenged this view, suggesting that the mammalian zygote may in fact be polarized and that the body axes are specified at the time of fertilization in a similar fashion to lower vertebrates. To date, no consensus has been reached, as different groups using different methodologies have results supporting both hypotheses.

In the mouse zygote, the position of the animal pole (marked by the second polar body), the sperm entry point (which triggers Ca^{2+} waves) or the plane defined by the two pronuclei in the zygote have been discussed as defining the plane of first cleavage. However, it is still unclear whether the position of these cues is directly responsible for the zygote polarity and subsequent position of the first cleavage plane. Alternatively, zygote polarity and the positions of the second polar body and the sperm entry point (and paternal pronucleus) might be determined by an intrinsic asymmetry already present in the oocyte.

It is still unsettled whether the fates of the two blastomeres are distinguishable and can be anticipated. The blastomere containing the sperm entry site generally divides first, and contributes preferentially to the embryonic region of the blastocyst, whereas its sister cell preferentially forms the abembryonic region. Parthenogenic eggs that do not contain a sperm entry point are able to divide and develop to blastocysts, although the two blastomeres do not tend to follow different fates. This indicates that, although during normal development the site of sperm penetration correlates with the later spatial arrangement of the blastocyst, it is not essential for patterning the embryo. Moreover, several studies have claimed that indeed the two blastomeres are similar, but it is the ellipsoidal shape of the zona pellucida that defines the embryonic-abembryonic axis. The topographic relationship between the blastocyst and the zygote remains a matter of debate. Although the blastocyst axes also correlate with the three axes of

the uterine horn, a relationship with the body axes of the future fetus is less clear.

Developmental Potency of the Early Mouse Embryo

In the mouse, both blastomeres of a two-cell stage embryo transplanted separately into foster mothers develop into identical mice. To assess the developmental potential of each blastomere of four-cell and eight-cell mouse embryos, Susan Kelly in 1977 combined isolated blastomeres with genetically distinguishable blastomeres of the same age, creating chimeric composites. Each blastomere was shown to contribute extensively to both embryonic and extraembryonic tissues (TE and visceral yolk sac), and to generate viable and fertile mice. This indicated that at these developmental stages all blastomeres are still totipotent. However, Andrzej Tarkowski and Joanna Wróbleswska in 1967 showed that isolated four-cell and eight-cell stage blastomeres were able to develop to blastocysts and implant, but were incapable of generating viable fetuses. This may be explained by the fact that a defined number of cell divisions (five) occurs before blastocyst formation. Thus, in contrast to the normal 32-cell blastocyst, isolated blastomeres from four-cell and eight-cell embryos resulted in 16-cell and eight-cell blastocysts, respectively. Tarkowski and Wróbleswska postulated that it is the position of a cell in the blastocyst that determines its fate: cells at the surface of the embryo become TE, whereas cells enclosed in the embryo become ICM. Blastocysts generated from isolated four- and eight-cell blastomeres contain progressively fewer cells in the ICM, making it likely that a minimum number of ICM cells are necessary for survival beyond the blastocyst stage.

Although different phenotypically, the two-cell subpopulations in the 16-cell morula are still plastic and able to produce cells of the other lineage, provided they are at the correct position in the embryo, that is, inside or at its surface. Cells of the ICM of 32- and 64-cell embryos are also still capable of contributing to all tissues of the conceptus (embryonic and extraembryonic), and are thus totipotent. The potency of TE cells has been difficult to determine because TE cells are not easy to isolate (tightly connected with each other) and because they are not readily integrated inside the embryo (low adhesiveness). After the 64-cell stage, the ICM loses totipotency.

Once the mouse embryo has implanted (up to embryonic day (E) 7.0), embryonic cells (including the primordial germ cells formed slightly later in development) lose their ability to contribute to the embryo when introduced directly into a host blastocyst to give rise to a chimeric embryo or chimera. In agreement, stem cells isolated from E5.5 and E6.5 embryonic (or epiblast) cells (EpiSC) are also unable to form chimeras. Remarkably, when introduced directly into genetically identical adult mice, epiblast cells are able to generate teratocarcinomas, tumors that contain tissues derived from the three germ layers (endoderm, mesoderm, and ectoderm) and an embryonal carcinoma (EC) stem cell population. EC cells are then able to form mouse chimeras when introduced into blastocysts suggesting that, although pluripotency is lost in the epiblast, it can be regained to a certain extent. Similarly, primordial germ cells isolated from E8.5 mouse embryos cultured to become embryonic germ (EG) cells and adult hematopoietic and neural stem cells are able to regain pluripotency, and can contribute to the embryo when introduced into blastocysts.

TABLE 13-1 Summary of Mouse and Human Pre-implantation Development

Stage (M)	Time (M)	Stage (H)	Time (H)	Developmental processes
Zygote	0–20 hr	Zygote 2-cell	0–60 hr	Axis determination?
2-cell	20–38 hr	4–8-cell	60–72 hr	Activation of embryo genome
4-cell	38–50 hr			
8-cell	50–62 hr	8–16-cell	~3.5 days	Compaction
16-cell	62–74 hr		~4.0 days	Two phenotypically different cells emerge
32-cell	~3.0 days	32-cell	~4.5 days	Blastocoelic cavity forms (cavitation)
64-cell	~3.5 days		~5.5 days	Blastocyst consists of two cell populations (ICM and TE)
128–256-cell	~4.5 days	166–286-cell	~6.0 days	Part of ICM differentiates to PrE; hatching, followed by implantation

During mouse and human development, the timing of each cleavage division is dependent on environmental factors (in vitro versus in vivo), individual variation, and mouse strain. The cleavage times presented here are ranges from several published sources. Adapted from Nagy et al. (2003), and Larsen (1997). hr, hours; H, human; ICM, inner cell mass; M, mouse; PrE, primitive endoderm; TE, trophectoderm

Genes Important During Pre-implantation Mouse Development

Before implantation, the embryo is relatively self-sufficient and can, for example, develop *in vitro* in simple culture media without growth factor supplements. Relatively few mutations (specific gene deletions, insertions, and more extensive genetic abnormalities) have been reported to result in pre-implantation lethality (Table 13-2). The reasons for this are not clear, but one may be that the initial presence of maternal transcripts in the zygote effectively results in maternal rescue. Ablation of specific maternal transcripts

TABLE 13-2 Lethal Mouse Mutations Affecting Differentiation During Early Development

Gene/Locus	Mutant Phenotype	References
Zar1	Zygote arrest	Wu *et al.* (2003) *Nat. Genet.*, *33*, 187
Dicer	Zygote arrest	Tang *et al.* (2007) *Genes Dev.*, *21*, 644
Hsf1	Zygote to 2-cell stage arrest	Christians *et al.* (2000) *Nature*, *407*, 693
Npm2	Zygote to 2-cell stage arrest	Burns *et al.* (2003) *Science*, *300*, 633
Mater (Nalp5)	2-cell stage arrest	Tong *et al.* (2000) *Nat. Genet.*, *26*, 267
HR6A	2-cell stage arrest	Roest *et al.* (2004) *Mol. Cell Biol.*, *24*, 5485
E-cadherin (uvomorulin, Cdh1)	2-cell stage arrest	Kanzler *et al.* (2003) *Mech. Dev.*, *120*, 1423
Padi6	2-cell stage arrest	Yurttas *et al.* (2008) *Development*, *135*, 2627
Basonuclin (Bnc1)	2-cell stage arrest	Ma *et al.* (2006) *Development*, *133*, 2053
Brg1	2- to 4-cell stage arrest	Bultman *et al.* (2006) *Genes Dev.*, *20*, 1744
Tcl1	4- to 8-cell stage arrest	Narducci *et al.* (2002) *PNAS*, *99*, 11712
Dppa3 (Stella, PGC7)	Failure to form blastocysts	Payer *et al.* (2003) *Curr. Biol.*, *13*, 2110; Bortvin *et al.*, (2004) *BMC Dev. Biol.*, *4*, 2
CTCF	Failure to form blastocysts	Wan *et al.* (2008) *Development*, *135*, 2729
Wt1 (Wilms' tumor 1)	Zygotes fail to undergo mitosis (background dependent)	Kreidberg *et al.* (1999) *Mol. Reprod. Dev.*, *52*, 366
Tgfb1	2- to 4-cell arrest (background dependent)	Kallapur *et al.* (1999) *Mol. Reprod. Dev.*, *52*, 341
C^{25H} *(pid)*	2- to 6-cell stage embryos fail to undergo mitosis	Lewis (1978) *Dev. Biol.*, *65*, 553
E-cadherin (uvomorulin, Cdh1)	Defects in compaction	Larue *et al.* (1994) *PNAS*, *91*, 8263; Riethmacher *et al.* (1995) PNAS, 92, 855
Trb (Traube)	Defects in compaction	Thomas *et al.* (2000) *Dev. Biol.*, *227*, 324
Mdn (morula decompaction)	Defects in compaction	Cheng & Costantini (1993) *Dev. Biol.*, *156*, 265
Om (ovum mutant)	Failure to form blastocysts (background dependent)	Wakasugi *et al.* (1967) *J. Reproduc. Fertil.*, *13*, 41; Baldacci *et al.* (1992) *Mamm. Genome*, *2*, 100
SRp20	Failure to form blastocysts	Jumaa *et al.* (1999) Curr. Biol., 9, 899
t^{12}, t^{w32}	Failure to form blastocysts	Bennett (1975) *Cell*, *6*, 441; Smith (1956) J. *Exp. Zool.*, *132*, 51
T^{hp} *(hairpin)*	Failure to form blastocysts	Babiarz (1983) *Dev. Biol.*, *95*, 342
Ts (Tail short)	Failure to form blastocysts	Paterson (1980) *J. Exp. Zool.*, *211*, 247
α-*E-catenin*	Failure to form blastocysts (TE defect)	Torres *et al.* (1997) *PNAS*, *94*, 901
Emi1	Failure to form blastocysts	Lee *et al.* (2006) *Mol. Cell Biol.*, *26*, 5373

TABLE 13-2 Continued

Gene/Locus	Mutant Phenotype	References
Vav	Blastocysts fail to hatch	Zmuidzinas *et al.* (1995) *EMBO J., 14*, 1
Os (oligosyndactyly)	Metaphase arrest at the early blastocyst stage	van Valen (1966) *J. Embryol. Exp. Morphol., 15*, 119; Magnuson & Epstein (1984) *Cell, 38*, 823
Brg1	Abnormal blastocyst development	Bultman *et al.* (2000) *Mol. Cell*, 6, 1287
A^x (lethal nonagouti)	Abnormal blastocyst development	Papaioannou & Mardon (1983) *Dev. Genet., 4*, 21
l(5)-1	Abnormal blastocyst development	Papaioannou (1987) *Dev. Genet., 8*, 27
t^{wPa-1}	Abnormal blastocyst development	Guenet *et al.* (1980) *Genet. Res., 36*, 211
PL16	Abnormal blastocyst development	Sun-Wada *et al.* (2000) *Dev. Biol.,228*, 315
CpG binding protein (CGBP)	Abnormal blastocyst development	Carlone & Skalnik (2001) *Mol. Cell Biol., 21*, 7601
Mbd3	Abnormal blastocyst development	Kaji *et al.* (2007) *Development,134*, 112
Thioredoxin (Txn)	Abnormal blastocyst development	Matsui *et al.* (1996) *Dev. Biol.,178*, 179
Gpt	Abnormal blastocyst development	Marek *et al.* (1999) *Glycobiology, 9*, 1263
Ltbp2	Abnormal blastocyst development	Shipley *et al.* (2000) *Mol Cell Biol* 20, 4879
Hba^{th-1}	Decreased TE cell number	Hendrey *et al.* (1995) *Dev. Biol.,172*, 253
Ay (lethal yellow)	Defects in TE formation	Papaioannou & Gardner (1979) *J. Embryol. Exp. Morphol., 52*, 153
Evx1	Defects in TE formation	Spyropoulos & Capecchi (1994) *Genes Dev.,8*, 1949
Eomes (Eomesodermin)	Defects in TE formation	Russ *et al.* (2000) *Nature, 404*, 95
Cdx2	Defects in TE formation	Chawengsaksophak *et al.* (1997) *Nature, 386*, 84
Egfr	Defects in ICM formation (background dependent)	Threadgill *et al.* (1995) *Science, 269*, 230
β1 integrin	Defects in ICM formation	Stephens *et al.* (1995) *Genes Dev., 9*, 1883; Fässler & Meyer (1995) *Genes Dev.,9*, 1896
Lamc1	Defects in ICM formation	Smyth *et al.* (1999) *J. Cell Biol.,144*, 151
B-myb	Defects in ICM formation	Tanaka *et al.* (1999) *J. Biol. Chem., 274*, 28067
Fgf4	Defects in ICM formation	Feldman *et al.* (1995) *Science,267*, 246
Fgfr2	Defects in ICM formation	Arman *et al.* (1998) *PNAS, 95*, 5082
Taube Nuss (Tbn)	Defects in ICM formation	Voss *et al.* (2000) *Development, 127*, 5449
Oct4 (Oct3, Pou5f1)	Defects in ICM formation	Nichols *et al.* (1998) *Cell, 95*, 379
Nanog	Defects in ICM formation	Mitsui *et al.* (2003) *Cell,113*, 631; Chambers *et al.* (2003) *Cell, 113*, 643
Eset	Defects in ICM formation	Dodge *et al.* (2004) *Mol. Cell Biol., 24*, 2478
Ronin	Defects in ICM formation	Dejosez *et al.* (2008) *Cell, 133*, 1162

The table is divided into three sections. The top lists maternal-effect genes. Embryos lacking these genes develop normally in heterozygous but not homozygous mothers. The middle section includes genes and loci that, when deleted, cause embryonic lethality before implantation. The lower section includes genes and loci that, when deleted, cause embryonic lethality during implantation but before the formation of the egg cylinder. Embryos deficient in most of these genes develop to normal blastocysts and are able to hatch and implant, but the whole embryo or selectively the TE or ICM (ICM- or primitive endoderm-derived cells) degenerates soon thereafter, leading to resorption. Extensive genetic abnormalities are not included in the table. ICM, inner cell mass; TE, trophectoderm

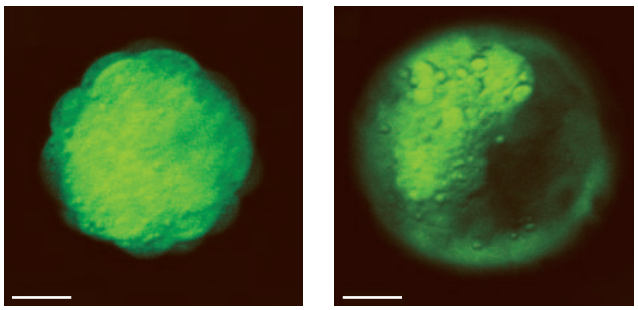


FIGURE 13-3 Oct4 expression at morula and blastocyst stages. GFP expression driven by distal elements of the Oct4 promoter (kindly supplied by H. Schöler) was used here to mimic endogenous Oct4 expression. In the morula, all blastomeres express high levels of Oct4 (A). In the early blastocyst, the inner cell mass expresses high levels of Oct4, whereas weaker expression is observed in trophectoderm cells (B).

in the zygote is not always feasible using conventional knockout techniques, because deficiency in candidate genes often results in lethality before adulthood. To date, only a limited number of maternal-effect genes involved in pre-implantation development have been identified (see Table 13-2).

Interestingly, most genes transcribed during pre-implantation development are detected immediately after genome activation and continue to be transcribed, resulting in mRNA accumulation. Therefore, to trigger the different specific developmental events during pre-implantation, post-transcriptional regulation may play an important role.

ES cells are derived from the ICM; therefore, it is not surprising that ES and ICM cells express common genes. Some of these genes have been described as being necessary for maintaining the undifferentiated phenotype of ES cells, and could be expected to play important roles in the segregation of the pluripotent ICM from the differentiated TE cell population. However, when deleted in the mouse, most of those genes appear to be crucial during implantation or gastrulation, but not during the pre-implantation period when both ICM and TE are formed. Most pertinent in this respect are the genes for leukemia inhibitory factor (LIF) and LIF receptors. Although mouse ES cells are highly dependent on LIF for maintenance of pluripotency in culture, deletion of neither receptor nor ligand genes appears to affect the pluripotency of the ICM at the blastocyst stage. Interestingly, *in vivo* LIF appears important for regulation of implantation (see the section on implantation).

The transcription factor Oct4 has the best-characterized involvement in regulating potency in mammals. Oct4 is initially expressed by all blastomeres, but expression becomes restricted to the ICM as the blastocyst forms (Figure 13-3). Thereafter, a transient up-regulation of Oct4 occurs in the ICM cells that differentiate to primitive endoderm. Interestingly, expression levels of Oct4 in mouse ES cells also regulate early differentiation choices, mimicking events in the blastocyst: mouse ES cells lacking *Oct4* differentiate to TE, whereas a twofold increase in Oct4 expression leads to endoderm and mesoderm formation. Mouse embryos deficient in *Oct4* are unable to form mature ICM and die around implantation time.

Other genes described as being involved in cell fate determination during pre-implantation development include *Taube nuss*, *B-myb*, *Nanog*, *Cdx2*, and *Eomes* (see Table 13-2). Both *Taube nuss* and *B-myb* homozygous-deficient mice develop to normal blastocysts. At the time of implantation, however, *Taube nuss*−/− ICM cells undergo massive apoptosis and the embryo becomes a ball of trophoblast cells; in *B-myb* knockout mice, the ICM also degenerates, but the reason for this is unclear. *Taube nuss* and *B-myb* seem to be necessary for ICM survival, whereas *Oct4* is required for establishment and maintenance of the ICM identity, but not cell survival. *Nanog* is expressed exclusively in the ICM and whereas *Oct4* prevents TE differentiation, *Nanog* prevents differentiation of ICM to primitive endoderm. In agreement, *Nanog*−/− blastocysts are formed, but the ICM in culture differentiates into endoderm. In contrast, *Cdx2* and *Eomes* appear to be involved in trophoblast development, and embryos lacking these genes die soon after implantation, probably because of defects in the trophoblast lineage.

FROM IMPLANTATION TO GASTRULATION

The mechanisms used by the mammalian embryo to implant are species-dependent, contrasting with the general developmental steps during the pre-implantation period. In addition, an intimate and highly regulated cross-talk between mother and mammalian embryo makes implantation a complex process.

Reaching the uterus, the blastocyst hatches from the zona pellucida and the TE cells become adhesive, expressing integrins that enable the embryo to bind the extracellular matrix (ECM) of the uterine wall. The mouse embryo adheres to the uterine wall via the mural TE cells of the abembryonic region and is slightly tilted. In contrast, human embryos bind through the embryonic region. Once attached to the uterus, trophoblast cells secrete enzymes that digest the ECM, allowing them to infiltrate and start uterine invasion. At the same time, the uterine tissues surrounding the embryo undergo a series of changes collectively known as the decidual response. These changes include formation of a spongy structure known as deciduum, vascular changes leading to the recruitment of inflammatory and endothelial cells to the implantation site, and apoptosis of the uterine epithelium.

The Murine Trophectoderm and Primitive Endoderm Cells

Apoptosis occurring in the uterine wall gives TE cells the opportunity to invade the deciduum by phagocytosing dead epithelial cells. At about E5.0, the mural TE cells cease division but continue endoreduplicating their DNA to become primary trophoblastic giant cells. This cell population is joined by polar TE cells that migrate around the embryo and similarly become polytene (secondary trophoblastic giant cells). However, other polar TE cells continue dividing and remain diploid, giving rise to the ectoplacental cone and the extraembryonic ectoderm which pushes the ICM into the blastocoelic cavity (Figure 13-4). TE cells, when cultured in the presence of fibroblast growth factor 4 (FGF4) and heparin, give rise to trophoblast stem (TS) cells, which are able to either self-renew or differentiate into trophoblastic giant cells. During implantation, the primitive endoderm layer forms two sub-populations: the visceral endoderm (VE) and the parietal endoderm (PE), both of which are extraembryonic tissues. The VE is a polarized epithelium closely associated with the extraembryonic ectoderm and the ICM-epiblast (Figure 13-4). When in culture, the primitive endoderm is able to give rise to a self-renewing stem cell population of extraembryonic endoderm (XEN) cells. Later in development, the VE contributes to the formation of the visceral yolk sac. PE cells migrate, largely as individual cells, over the TE (Figure 13-4) and secrete large amounts of ECM to form a thick basement membrane known as Reichert's membrane. The PE cells, together with the trophoblastic giant cells and Reichert's membrane, form the parietal yolk sac.

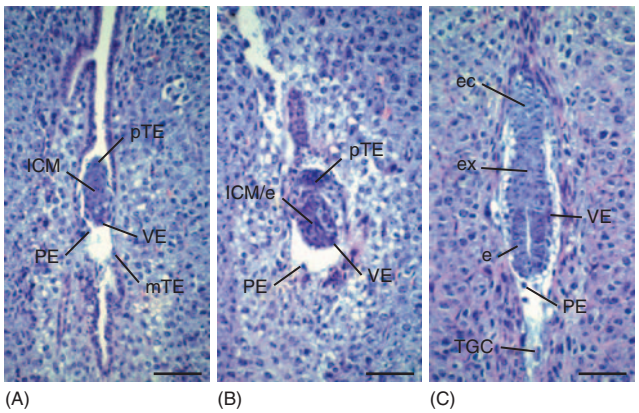


FIGURE 13-4 Tissue formation and movements during and shortly after implantation of the mouse embryo (E5.0–E5.5). During implantation, the cell division rate in the embryo increases, leading to rapid growth (A–C). The primitive endoderm cells segregate into visceral endoderm (VE) and parietal endoderm (PE). The polar trophectoderm cells (pTE) form the ectoplacental cone (ec) and the extraembryonic ectoderm (ex). pTE cells, together with mural trophectoderm cells (mTE), contribute to form the trophoblastic giant cells (TGC). The inner cell mass (ICM) cavitates and organizes into an epithelium known as the epiblast (e).

Development of the Murine Inner Cell Mass to the Epiblast

The ICM located between the recently formed extraembryonic ectoderm and the VE gives rise to all cells of the embryo proper. During implantation, the ICM organizes into a pseudostratified columnar epithelium (also referred to as primitive or embryonic ectoderm, epiblast, or egg cylinder) surrounding a central cavity, the proamniotic cavity (Figure 13-4). Signals from the VE, including BMPs, are responsible for apoptosis in the core of the epiblast leading to its cavitation. Between E5.5 and E6.0, the proamniotic

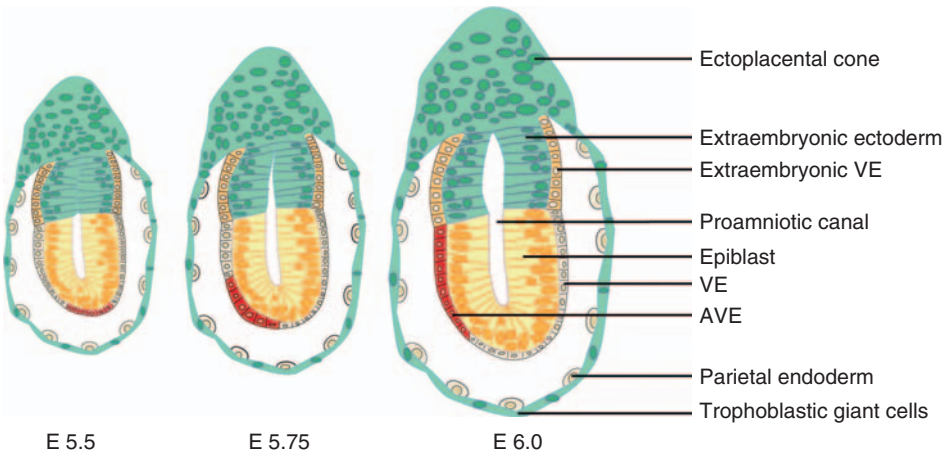


FIGURE 13-5 Tissue formation and movements in the pregastrulation mouse embryo (E5.5–E6.0). During this period, the extraembryonic ectoderm organizes into an epithelium. The proamniotic cavity, initially restricted to the epiblast, now expands into the extraembryonic ectoderm, forming the proamniotic canal. At E5.5, the most distal visceral endoderm cells (red) express a different set of markers than the surrounding visceral endoderm (VE). These distal VE cells move from the distal tip to surround the prospective anterior part of the epiblast and form the anterior visceral endoderm (AVE). The VE surrounding the extraembryonic ectoderm consists of a columnar epithelium, whereas the VE cells surrounding the epiblast are more flattened. Adapted from Lu *et al.*, 2001.

cavity expands to the extraembryonic ectoderm, forming the proamniotic canal (Figure 13-5).

After implantation, a wave of *de novo* DNA methylation occurs, leading to epigenetic reprogramming (finished by E6.5). This affects the entire genome to a different extent in embryonic and extraembryonic lineages, and may be responsible for the observed loss of ability to contribute to chimeras. After implantation, the rate of cell division increases, followed by rapid growth. At E4.5, the ICM consists of approximately 20 to 25 cells, at E5.5 the epiblast has about 120 cells, and at E6.5 it consists of 660 cells.

At E6.5 the embryo is not cylindrical, but already has a long and a short axis. Gastrulation starts with the formation of a morphologically visible structure (the primitive streak) at one side of the short axis, which becomes the posterior side of the embryo. During gastrulation, the embryo undergoes an apparent shift in orientation inside the deciduum, the three definitive germ layers are formed, the germline is set aside, and the extraembryonic mesoderm that contributes to the visceral yolk sac, placenta, and umbilical cord is generated. An overview of tissue formation and movement during mouse gastrulation is shown in Figure 13-6.

The Human Embryo

Human development during implantation and gastrulation is significantly different from that of the mouse. Briefly, the human trophoblast cells invade the uterine tissue and form the syncytiotrophoblast, a tissue similar to the mouse giant trophoblast cells. However, the trophoblast cells that contact the ICM and the blastocoelic cavity remain single cells and diploid, and are known as cytotrophoblasts. These cells proliferate and fuse with the syncytiotrophoblast. In humans, no structure equivalent to the murine extraembryonic ectoderm is formed.

Human primitive endoderm cells, also known as hypoblast cells, form on the surface of the ICM and proliferate. Some of these cells migrate to line the blastocoelic cavity, leading to the formation of the exocoelomic membrane and the primary yolk sac. The human primary yolk sac is not equivalent to the murine parietal yolk sac, although both are transient structures. Moreover, it is still unclear whether human embryos develop a PE-like cell type. Paralleling the formation of the murine Reichert's membrane, a spongy layer of acellular material known as the extraembryonic

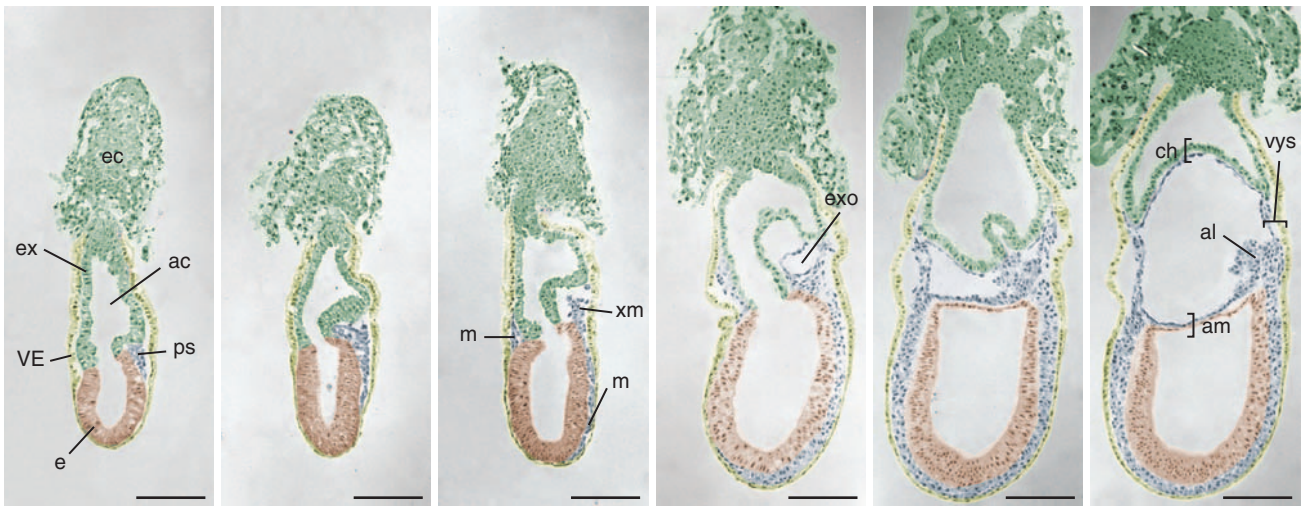


FIGURE 13-6 Tissue formation and movements during the gastrulation of the mouse embryo (E6.5–E7.5). Gastrulation begins with the formation of the primitive streak (ps) in the posterior side of the E6.5 embryo at the junction of the extraembryonic ectoderm (ex) and epiblast (e) (A). As more cells ingress through the streak, it elongates towards the distal tip of the embryo, between epiblast and visceral endoderm (VE) (B). While the newly formed embryonic mesoderm (m) moves distally and laterally to surround the whole epiblast, the extraembryonic mesoderm (xm) pushes the extraembryonic ectoderm upwards and to the center (C, D). The extraembryonic mesoderm develops lacunae, creating a mesoderm-lined cavity known as the exocoelom (exo). The exocoelom enlarges and, as a consequence, the tissue at the border of extraembryonic and embryonic ectoderm fuses, dividing the proamniotic cavity (ac) in two and forming the amnion (am) and the chorion (ch) (E). The layer of extraembryonic mesoderm and the visceral endoderm together form the visceral yolk sac (vys). At the posterior side of the embryo the allantois (al) and the primordial germ cells are formed (E, F). The tissues colored green (the extraembryonic ectoderm and ectoplacental cone (ec)) are derived from the trophectoderm. The tissues in yellow are derived from the primitive endoderm and epiblast cells that passed through the streak, generating the definitive endoderm. These definitive endoderm cells intercalate with the visceral endoderm in the region of the streak, but also form a larger patch of cells at the distal part of the embryo. This patch of cells moves anteriorly, displacing the anterior visceral endoderm, which moves towards the extraembryonic region of the embryo. The tissues colored orange are derived from the inner cell mass and remain ectoderm. The tissues colored blue are formed during gastrulation and represent primitive streak and mesoderm-derived tissues (excluding the primordial germ cells present at the basis of the allantois). For the lineages of early mouse development see Figure 13-2.

reticulum is formed between cytotrophoblast and the exocoelomic membrane. Thereafter, the extraembryonic reticulum is invaded by extraembryonic mesoderm. The origin of this tissue in humans is still unknown. The extraembryonic mesoderm proliferates to line both Heuser's membrane and cytotrophoblast. The extraembryonic reticulum then breaks down, and is replaced by a fluid-filled cavity, the chorionic cavity.

A new wave of hypoblast proliferation generates cells that contribute to the formation of the definitive yolk sac. This new structure displaces the primary yolk sac, which buds off and breaks up into small vesicles that remain present in the abembryonic pole. The definitive yolk sac in humans is equivalent to the visceral yolk sac in the mouse.

The human ICM organizes into a pseudostratified columnar epithelium and cavitates, producing the amniotic cavity. The ICM cells that lie on the hypoblast are known as the epiblast, and give rise to the embryo proper. The ICM cells that contact the trophoblast form the amnion. The human embryo forms a bilaminar embryonic disc, similar to chick embryos, and the patterns of cell movement during gastrulation are conserved between chick and humans.

With such diversity in extraembryonic structures supporting the development of the ICM in mice and humans, it is not surprising that ES cells derived from mice and humans are not equivalent. They differ in developmental potency, for example, in their ability to differentiate to TE. Human ES cells can form TE in culture, but under normal circumstances mouse ES cells do not. Furthermore, mouse ES cells in culture have recently been shown to develop into cells with primordial germ cell properties. In turn, these cells can develop into sperm- or oocyte-like cells. The potential of these cells to fertilize or to be fertilized and generate viable mice is still unknown. It is not yet known whether human ES cells have this potential to form mature gametes in culture. Mouse and human ES cells also express different cell surface markers, have different requirements in culture for self-renewal, and respond differently to growth and differentiation cues, the most striking being the response to LIF.

Recent literature has shown that mouse ES cells are a heterogeneous population in terms of the expression of markers, containing cells that resemble either ICM or epiblast cells. The mouse ES cells most similar to epiblast cells resemble mouse EpiSCs, but are not equivalent. In contrast to mouse ES cells, EpiSCs very closely resemble human ES cells. Current thoughts suggest that the unusual characteristics of mouse ES cells arise from the fact that the mouse uses a reproductive strategy known as facultative embryonic diapause. This means that the blastocyst has the capacity to wait in the uterus until the conditions for implantation become favorable (for example if the female is still lactating). In humans and most other mammals, this is not the case and the embryo either implants and develops or degenerates.

Implantation: Maternal Versus Embryonic Factors

In mice, the presence of the blastocyst in the uterus is sufficient to trigger ovarian production of progesterone and estrogen. These two hormones are absolutely required for embryo survival, because they prime the uterus for implantation and decidualization. The uterus starts producing LIF and members of the epidermal growth factor (EGF) family, including EGF, heparin-binding EGF, transforming growth factor-alpha (TGFα), and amphiregulin. Those molecules, together with Hoxa10, induce the production of cyclooxygenase (COX) enzymes, the rate-limiting enzymes in the production of prostaglandins. The embryo, on the other hand, also produces important molecules that act in autocrine and paracrine ways. With a few exceptions, all of these factors and corresponding receptors play crucial roles during this period, and when deleted in the mouse leads to lethality during or soon after implantation.

The suppression of the maternal immune response is also essential during implantation, but is still incompletely understood. TE cells, the only cell population of the conceptus that physically contacts maternal cells, have developed several mechanisms to avoid rejection. Examples are the production of numerous factors and enzymes, including indoleamine 2,3-dioxygenase (IDO) by the TE cells that suppress the maternal immune system and the lack of polymorphic class I and II major histocompatability complex (MHC) antigens in TE cells.

The Role of Extraembryonic Tissues in Patterning the Mouse Embryo

Extraembryonic tissues are not only necessary for nutrition and regulating implantation during development, but also play crucial roles in patterning the embryo before and during gastrulation. Unequivocal evidence for this role comes from the analysis of chimeric embryos generated from blastocysts colonized with ES cells. In chimeras, ES cells preferentially colonize epiblast-derived tissues. It is, therefore, possible to generate embryos with extraembryonic tissues of one genotype and epiblast-derived tissues of another genotype. For example, nodal is expressed embryonically and extraembryonically (depending on the developmental stage). Furthermore, *nodal*-deficient embryos fail to gastrulate. It was thus initially difficult to distinguish embryonic from extraembryonic functions. However, when *nodal*−/− ES cells were introduced into wild-type blastocysts, the extraembryonic tissues were wild-type, whereas epiblast-derived tissue lacked *nodal*. The developing chimera was essentially normal until midgestation, suggesting that the presence of *nodal* (exclusively) in the extraembryonic tissues is sufficient to rescue embryonic patterning.

In contrast to the extensive mixing of epiblast cells, labeled primitive endoderm cells develop as more coherent clones, consistent with the function of the VE in embryo patterning. The primitive endoderm cells in the vicinity of the second polar body preferentially form VE cells surrounding the epiblast, whereas cells away from the second polar body preferentially form VE cells surrounding the extraembryonic ectoderm.

At E5.5, the most distal VE cells are characterized by the expression of the homeobox gene *Hex*. This cell population migrates toward the prospective anterior side of the embryo during the next day of development, producing an endodermal stripe known as the anterior visceral endoderm (AVE) (Figure 13-5). The AVE is therefore the first clear landmark of an anterior–posterior axis in the embryo, preceding the formation of the primitive streak, at the opposite side of the embryo during gastrulation.

Before gastrulation, the extraembryonic ectoderm signals to the proximal epiblast, inducing expression of several genes important for posterior proximal identity. In contrast, signals from the distal VE (and later the anteriorly migrating AVE) seem to inhibit the expression of that same set of genes, restricting in this way the posterior fate.

Both VE and VE-like cell lines secrete signals that are able to induce differentiation of mouse and human ES cells at least toward cardiomyocytes. Making use of the tissues or sequences of signal transduction pathways used by the embryo for its own patterning and differentiation seems to be one efficient way to direct ES cell differentiation. It is therefore paramount to understand the events that take place early during embryonic development to define differentiation signals more precisely.

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KEY WORDS

Blastocyst Very early animal embryo consisting of a spherical outer epithelial layer of cells known as the trophectoderm (that forms the placenta), a clump of cells attached to the trophectoderm known as the inner cell mass (from which stem cells are derived), and a fluid-filled cavity, the blastocoel. When fully expanded, the blastocyst "hatches" from the zona pellucida in which it has developed and implants in the uterus of the mother.

Chimera Organism made up of cells from two or more different genetic donors. In general, two genetically different very early embryos (at the morula stage) are aggregated to form one single embryo or, alternatively, embryonic stem cells are allowed to attach to a morula or are injected inside a host blastocyst. Embryonic stem cells integrate preferentially in embryonic tissues, whereas the extraembryonic tissues are derived from the recipient embryo.

Differentiation Process during embryogenesis or in pluri/multipotent cells in culture in which an increase in the complexity or organization of a cell or tissue results in a more specialized function.

Implantation Process by which the mammalian blastocyst physically connects with the uterus of the mother. It involves the displacement of the uterine epithelium cells, and extracellular matrix degradation by proteolytic enzymes secreted by the embryo. Implantation occurs only in mammalian development and is necessary to provide the growing embryo with sufficient protection and metabolic needs.

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Chapter 14

Primordial Germ Cells in Mouse and Human

Anne McLaren and Susana M. Chuva de Sousa Lopes

INTRODUCTION

What are Primordial Germ Cells?

The germ cell lineage terminates in the differentiation of the gametes (eggs and spermatozoa). In mammals the lineage arises in the extraembryonic mesoderm at the posterior end of the primitive streak. From here the germ cells migrate to the two genital ridges, which later form the gonads. During this period, they proliferate at a steady rate and are known as primordial germ cells (PGCs). Once in the genital ridges, they may be termed gonocytes. Proliferation ceases in the male genital ridge when the germ cells transiently arrest in G0/G1 as prospermatogonia, and in the female genital ridge when they enter prophase of the first meiotic division as oocytes. These events, which occur before birth in both mouse and human, mark the initiation of the lengthy processes of spermatogenesis and oogenesis, respectively, and the end of the primordial phase of germ cell development.

Primordial Germ Cells Are Not Stem Cells

Stem cells are commonly defined as cells with a choice: they can divide to form either two cells like themselves (self-renewal) or one cell like themselves and one that is embarking on a pathway of differentiation (asymmetric division). PGCs do not at any stage constitute a stem cell population: each of the cell divisions that they undergo (9 or 10 in the mouse, more in the human) moves them further along their developmental trajectory. At a later stage in the male germ cell lineage, a true stem cell population forms: spermatogenic stem cells in the testis divide slowly to form a self-renewing population throughout the lifetime of the male, giving rise to waves of proliferating spermatogonia that enter meiosis as spermatocytes, then differentiate into spermatids, and finally become mature spermatozoa. PGCs can give rise *in vitro* to pluripotent stem cell populations that will proliferate indefinitely, given appropriate culture conditions, but PGCs *in vivo* are not stem cells.

ORIGIN OF THE GERM CELL LINEAGE

Identification of Primordial Germ Cells

In many mammals, including mouse and human, PGCs can be readily identified by staining for alkaline phosphatase activity. Tissue nonspecific alkaline phosphatase (TNAP) is expressed in many tissues, but activity is markedly higher in germ cells than in the surrounding somatic cells throughout the primordial period. The function of the enzyme is not clear, because it has been shown that disruption of the *Tnap* gene does not appear to affect germ cell development. PGCs have also been identified histologically and by electron microscopy and, more recently, also by a variety of genetic markers (see the section on gene expression below). The allantois, once it has started to grow, forms a convenient landmark for spotting the cluster of PGCs at its base (about eight days postcoitum (dpc) in the mouse). A cluster of about 45 cells could first be identified, in the same location, but before an allantois was apparent, at 7.25dpc, in mid-gastrulation.

Early Studies

The failure to identify germ cells in the mouse embryo by morphology, alkaline phosphatase activity, or any other feature at any stage of development earlier than mid-gastrulation was frustrating because, in many invertebrates and lower vertebrates, the germline could be traced back to very early development, even to the egg. In *Drosophila melanogaster* the germline derives from the pole plasm, incorporated

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in the pole cells, the first cells to form; in *Caenorhabditis elegans* the germ cell determinants (P granules) present in the egg are asymmetrically segregated during the first four cell divisions, to form the germline; in *Xenopus laevis* and other anuran amphibia, the germ plasm apparent in the egg can be followed into the germ cells as they form. Expression of homologs of the germline-specific gene *Vasa* is localized in "germ plasm" from early cleavage stages onward in both zebra fish and chick. No such lineage could be detected in the mouse or any other mammal. Chimera studies were unable to identify any cells in the pre-implantation embryo that were uniquely associated with the germline. Circumstantial evidence had led some early workers to assert that the germ cell lineage in mammals had an extraembryonic origin. Subsequently, however, various lines of evidence, in particular the transplantation studies of Gardner and Rossant (1979), established beyond doubt that the ancestors of the germ cell lineage were to be found in the epiblast, which is derived from the inner cell mass of the blastocyst, not from the outer trophectoderm.

Time and Place of Lineage Determination

Fate-mapping of the mouse epiblast has been achieved by injecting a long-lasting fluorochrome into single epiblast cells of embryos removed from the uterus in early gastrulation. Subsequent culture of the embryo for 48 hours reveals the tissue fate of the clonal descendants of the injected cell. The study of Lawson and Hage (1994) established that only the most proximal cells of the epiblast, those close to the extraembryonic ectoderm, included PGCs among their descendants. No clone consisted only of PGCs, proving that at the time of injection (6.0 or 6.5dpc), the injected cells were not lineage-restricted. Indeed, PGCs never constituted more than a small proportion of each marked clone, and marked PGCs only made up a small proportion of the total number of TNAP-positive PGCs. Clonal analysis revealed that the most likely time of germ cell specification was 7.2dpc, approximately the time at which a TNAP-positive cluster of putative germ cells had first been identified in the extraembryonic mesoderm.

Clones without any PGCs contained more cells than those with PGCs, and the fewer the PGCs the larger was the total number of cells. This suggested that germ cell determination was associated with an increase in cell doubling time, to about 16 hours for PGCs, in contrast to six to seven hours for the surrounding somatic extraembryonic mesoderm cells. Counts of PGCs on successive days had previously given a similar figure of 16 hours for PGC doubling time in the 8.5–13.5dpc period.

A recent study from Surani's group has broadened our understanding of germ cell determination considerably. They identified a population of about six epiblast cells expressing the transcriptional repressor *Prdm1* (or *Blimp1*) as early as 6.25dpc in the future posterior proximal site of the embryo just before gastrulation. From 6.5–7.2dpc more epiblast cells express *Prdm1* and at E7.2dpc all *Prdm1*-positive epiblast cells (about 45 cells) undergo specification. After this event no more cells enter the germ cell lineage. *Prdm1*-expressing PGC progenitor cells are indeed lineage-restricted in the sense that they only give rise to PGCs if allowed to develop normally.

In amphibia, the germ cell lineage can be traced back to the egg in frogs and toads (anura), but in urodeles it is induced in mid-gastrulation, in the mesoderm, similar to mammals. A. D. Johnson has proposed that the urodele-mammal mode of development is the more primitive. The early-determination mode, characterized by germ plasm, must then have been derived several different times in evolution. This view is supported by the expression pattern of germline-specific genes such as *Vasa* and *Dazl*, which are similar in urodeles and mammals and also in primitive fishes, but very different in anura, chick, zebra fish, fruitfly, and worm.

Signaling Factors

Bmp4 homozygous mutants die around the time of gastrulation. Using a strain combination that survived until late gastrulation, Hogan's group showed that the homozygotes had no allantois and no PGCs. BMP4 protein, known to act as a signaling molecule, is normally expressed in the extraembryonic ectoderm (Lawson *et al.*, 1999). Chimeras made between normal embryonic stem (ES) cells and *Bmp4*-negative embryos, in which the epiblast contained both cell types but the extraembryonic tissues (which do not incorporate ES cell derivatives) were all BMP4-negative, also lacked both allantois and PGCs. Hence, *Bmp4* expression in the extraembryonic ectoderm is required for the establishment of the germ cell lineage, implicating a *Bmp4*-dependent signal to the immediately adjacent proximal epiblast cells. *Bmp8b* and *Bmp2* mutants have a reduced number of PGCs, suggesting that BMP8b (produced by the extraembryonic ectoderm) and BMP2 (produced by the visceral endoderm) are also involved in signaling, perhaps interacting with BMP4. The molecular details of the signaling pathway have still to be established.

Further evidence that signaling from the extraembryonic ectoderm and visceral endoderm are required for germ cell determination comes from experiments in which distal epiblast cells, which would normally give rise to neurectoderm, were transplanted at 6.5dpc to a proximal location. Some of the PGCs that formed expressed the genetic marker carried by the donor embryo. This finding suggests that all epiblast cells in early gastrulation have the potential to develop into PGCs, if they receive the appropriate signals. Signals emanating from the extraembryonic ectoderm and visceral endoderm predispose the neighboring posterior proximal epiblast cells to a germ cell fate, probably by inducing the expression of *Prdm1*, but they do not determine germ cell fate, because the clonal analysis referred to in the previous

section on time and place of lineage restriction showed that, for some epiblast cells, only a small proportion of their descendants became PGCs. In agreement, expression of *Prdm1* is necessary but not sufficient to ensure germ cell determination. Once the cells have passed through the primitive streak and reached the cluster region, a further signal or signals may be required to complete the specification process and halt further movement. This further signal or signals may have origin in the extraembryonic mesoderm.

Gene Expression

For decades, the high level of expression of *Tnap* was the only useful genetic marker for the initial stages of the mammalian germ cell lineage. *Oct4* is expressed diffusely during gastrulation, so PGCs are not distinguished from the surrounding tissues until about 8.0dpc. Other useful PGC markers (*SSEA1*, *mouse vasa homolog*, *Dazl*, *Nanog*) are expressed somewhat later.

According to the study of Saitou *et al.* (2002), in which single-cell cDNA libraries were made from the cluster region of 7.0–7.5dpc mouse embryos, PGC libraries could be distinguished from somatic cell libraries by the relatively high level of expression of *Tnap* and the absence of expression of *Hoxb1*. Two new germ cell-specific genes were isolated from the PGC libraries. These were termed *fragilis* (or *Ifitm3/Mil1*), and *stella* (or *Dppa3/Pgc7*). From about 6.0dpc on *fragilis* is expressed in the proximal region of the epiblast. Its expression pattern shifts as the epiblast cells move towards the primitive streak, and by 7.2dpc it is concentrated around the cluster region of *Prdm1*-positive PGC progenitors. Once the PGCs begin to move away, *fragilis* is down-regulated, although it comes on again later. It belongs to a widely-distributed family of interferon-inducible genes, of which other members code for proteins showing homotypic adhesion and changes in cell cycle regulation. In contrast, *stella* is a novel gene, not part of a gene family. It carries a nuclear-localization signal. *stella* is up-regulated in the cluster region, where *fragilis* expression is strongest, at the time when PGCs can first be visualized by TNAP-activity. *stella* maintains its PGC-specific expression during germ cell migration and into the genital ridges. Thus, it provides a valuable new germ cell-specific genetic marker. In contrast to *Prdm1*, but similarly to *Tnap*, both *fragilis* and *stella* are not necessary for PGC determination.

MIGRATION

Route

As the germ cell cluster breaks up, at about 8.0dpc, the PGCs appear to migrate actively into the visceral endoderm, which carries them along as it invaginates to form the hind gut. Although initially ventral, they become distributed around the hind gut and pass dorsally into the genital ridges (gonad primordia), through the body wall, or up the dorsal mesentery and round the coelomic angle at 10.0–11.0dpc. The entire period of migration has been visualized by Wylie's group on a video of PGCs carrying a transgenic green fluorescent protein marker. The PGCs show locomotory behavior throughout, which is nondirectional in the wall of the hind gut where they are carried along passively, but strongly directional as they leave the hind gut and enter the genital ridges. Once in the ridges, the locomotory behavior ceases.

Guidance Mechanisms

Little is known of the mechanism that encourages the PGCs to enter the visceral endoderm (rather than the allantois or some other extraembryonic mesodermal region) as the cluster breaks up. The interplay between the expression of E-cadherin and N-cadherin in the cluster, as well as the differential expression of *fragilis*-family members is probably involved in this process. Contact guidance may play a part in facilitating exit from the endodermal hind gut wall, because the adhesion properties (particularly to laminin) change at this time. As the PGCs move toward the genital ridges, they have been reported to contact one another through long cellular processes, forming a loose network. The molecular nature of the directional signals that attract them toward the genital ridges has yet to be definitively established, but in mice as well as in zebra fish there is evidence that the chemokine–receptor pair stromal cell-derived factor 1 (SDF-1) and its G-protein-coupled receptor (CXCR4) act to guide migrating PGCs.

Gene Expression

Certain gene products need to be present if PGC migration is to take place normally. Abnormal migration patterns are readily detected because they result in subfertility or sterility. Two classic mouse mutants, *White-spotting* and *Steel*, both show sterility in the homozygous condition, and defects in hematopoiesis and pigment cell migration. *White-spotting* (*W*) codes for c-kit, a cell surface receptor; *Steel* (*SL*) codes for its ligand, stem cell factor. If either element of this signal transduction pathway is defective, PGC migration is disturbed, proliferation is affected, programmed cell death (apoptosis) occurs, and few germ cells reach the genital ridges. Other genes which are known to be required for normal PGC migration include *gcd* (*germ cell deficient*), *β1 integrin*, TGFβ, and *Fgf8*.

GERM CELLS IN THE GENITAL RIDGE

Phenotype

Mouse PGCs migrate actively into the genital ridges between 10.5 and 11.5dpc. Soon after entry into the ridges,

their motile phenotype changes to a rounded shape, they up-regulate E-cadherin, movement ceases, and they form loose groups. Gene expression changes; *mvh* (*mouse vasa homolog*) and *gcna* (*germ cell nuclear antigen*) act as useful germ cell markers at this stage. Proliferation continues for another couple of days, still with a doubling time of about 16 hours. However, by 12.5dpc, germ cells in both female and male embryos are entering the premeiotic cell cycle, mitosis ceases, and meiotic genes such as *Scp3* (*Synaptonemal complex protein 3*) are up-regulated.

A detailed quantitative study of germ cells in the human fetal ovary, before and during entry into meiosis, was made by Baker (1963). In contrast to the mouse, where the number of oocytes present in the ovary remains constant until the time of birth, the number of human oocytes in the ovary increases drastically between the second and the fifth month of pregnancy and decreases from the fifth month of pregnancy until birth. Germ cells in mitosis are observed until the seventh month of pregnancy, and coexist with oocytes that have entered meiosis. The passage through the successive stages of meiotic prophase is also not well-synchronized in humans. As a consequence, germ cells expressing pre-meiotic and post-meiotic genes can be observed in the same ovary.

Sex Determination

In mouse female genital ridges, the germ cells proceed at about 13.5dpc into prophase of the first meiotic division, passing through leptotene, zygotene, and pachytene stages before arresting in diplotene, in primordial follicles, shortly after birth. Germ cells enter into first meiotic prophase at the same time in ectopic locations outside the genital ridge, such as in the adrenal primordium, in male and in female embryos. Germ cells isolated from both male and female embryos at 11.5dpc or earlier will also enter meiosis at this time in cultured aggregates of lung tissue, or even on a feeder layer *in vitro*. Recent data suggests that retinoic acid (produced *in vivo* by the mesonephros in both sexes) is responsible for the induction of meiosis in the ovary.

In contrast, in the male genital ridges the germ cells at about 13.5dpc down-regulate meiotic genes such as *Scp3* and enter mitotic arrest in G_1/G_0 as prospermatogonia. This change in cell fate determination is not cell autonomous, but is induced by supporting somatic cells in the male genital ridge. The somatic signal that blocks entry into meiosis is probably *Cyp26b1*, which encodes for an enzyme that degrades retinoic acid. Expression of *Cyp26b1* in the early gonadal ridges of both sexes may prevent PGCs from entering meiosis when they arrive there. In XY embryos, the testis-determining gene on the Y-chromosome (*Sry*) is expressed by the supporting somatic cells for about 36 hours, from 10.5 to 12.0dpc. *Sry* seems to be responsible for the upregulation of *Cyp26b1*. *Sox9*, another presumed target of *Sry*, is expressed from 11.5dpc on and is required for the differentiation of Sertoli cells from the supporting somatic cell lineage, and for their subsequent maintenance. By 12.5dpc, germ cells in the male genital ridge are enclosed in cords, lined with Sertoli cells, and surrounded by peritubular myoid cells that have migrated in from the mesonephric region in response to a *Sry*-dependent signal. In agreement, germ cells removed from a female genital ridge at 12.5dpc or earlier and aggregated with somatic tissue from a 12.5dpc male genital ridge will enter mitotic arrest rather than meiotic prophase, but by 13.5dpc they are already committed to the female pathway of development. The male germ cells, however, are already committed to the male pathway by 12.5dpc and develop as prospermatogonia even when aggregated with female genital ridge cells.

X-chromosome Reactivation

In female mammals, one or other of the two X-chromosomes, at random, is inactive during gastrulation in all cells of the somatic lineages, and also in the germ cell lineage, and therefore all embryonic cells have just a single X-chromosome active (dosage compensation). During germ cell migration, female PGCs lose the characteristic marks on the silent X (Xi) and the transcriptional reactivation of the Xi begins. This early phase of Xi reactivation may be intrinsically programmed in PGCs. Once in the genital ridges, both wide transcriptional and translational activity is detected in human and mouse oocytes. In agreement, when 9.5dpc migratory PGCs were co-cultured with 11.5dpc female XX genital ridges, translational reactivation of the Xi was observed within 48 hours. This translational reactivation was not observed when 9.5dpc PGCs were cultured alone or together with 11.5dpc male XY genital ridges. Both X-chromosomes remain active throughout oogenesis. Reactivation occurs not only in the female genital ridge, but also in XX germ cells developing in a phenotypically male XX genital ridge. *Xist* is involved in the initiation of X-chromosome inactivation. It produces a *Xist* stable non-coding RNA transcript that coats the whole chromosome and can be visualized by RNA fluorescent *in situ* hybridization (FISH).

Epigenetic Changes

Patterns of DNA methylation are imposed during gastrulation on the nascent somatic cell lineages but not on PGCs, perhaps because of their extraembryonic birthplace. Global DNA methylation in PGCs is still further decreased once the germ cells are in the genital ridges. Imprinted genes (i.e., genes in which only the paternal or only the maternal allele is expressed, but not both) are characterized by differential DNA methylation at specific CpG sites. Genes shown to be imprinted in the mouse are not necessarily imprinted in the human (e.g., *Igf2r*). In the germ cell

lineage, the previous genomic imprint has to be erased, and a new imprint is established according to the sex of the embryo. The differential site-specific methylation in some imprinted genes has been examined in mouse germ cells by bisulfite sequencing. It decreases during PGC migration or shortly after entry into the genital ridges. At 10.5dpc, some site-specific methylation is present, much less at 11.5dpc, and very little at 12.5dpc. New imprints are established before or after birth: in the female germline, for example, different imprinted genes acquire their new methylation pattern at different stages of oogenesis.

DNA methylation is an important mechanism to silence transposons in the mammalian germline. The small RNA signaling pathway (piRNAs and microRNAs) may be linked to the DNA methylation of retrotransposons in germ cells. The deletion of molecules involved in the synthesis of both piRNAs and miRNAs in mice results in spermatogenesis defects.

In addition to the epigenetic changes in DNA methylation, the pattern of histone modifications also changes gradually but profoundly from the time of PGC specification until their arrival in the genital ridges. In particular around 11.5dpc, PGCs undergo major changes in nuclear architecture and chromatin composition, including the erasure of several histone modifications and the exchange of histone variants. It is unclear whether this is linked to a DNA repair-mechanism.

EMBRYONIC GERM CELLS

Embryonic Germ Cell Derivation

Early attempts to establish long-term cultures of mouse PGCs met with little success, even when feeder cells were used. Chromosomally stable stem cells were eventually obtained by combining three growth factors in the cultures: stem cell factor (SCF), fibroblast growth factor 2 (bFGF), and leukemia inhibitory factor (LIF). These stem cells were termed embryonic germ cells (EGCs) to distinguish them from embryonic stem cells (ESCs). EGCs, like ESCs, proved capable of indefinite proliferation in culture and were pluripotent both *in vitro* and *in vivo* in chimeras in which they colonized all cell lineages including the germline. EGC lines have been derived from PGCs before and during migration (8.5, 9.5dpc), and also from PGCs in the genital ridge but only up to 13.5dpc. EGC lines have also been derived in some other mammalian species, including from human PGCs. Like mouse EGCs, human EGCs show high levels of alkaline phosphatase activity and express SSEA1.

Xist Expression

In PGCs, *Xist* may code for a transcript (migrating XX PGCs), or expression may be entirely absent (XY PGCs; XX PGCs after X-chromosome reactivation in the genital ridge). Undifferentiated EGC lines are always characterized by a weak *Xist* transcript, visualized by RNA FISH as a small dot overlying the locus. A similar situation is seen in ESCs. Once the EGCs or ESCs start to differentiate, the small dot on the *Xist* locus disappears and is replaced by an enrichment of *Xist* transcript on the silent X-chromosome or absence of expression, according to whether the cell is XX or XY. In human EGCs the expression of *Xist* has not been analyzed, but the expression of *Xist* in female human ESCs is variable and depends on the cell line and passage number.

Epigenetic Changes

The epigenetic status of imprinted genes in EGCs might be expected to reflect that in the PGCs from which they were derived. However, this is not so. Site-specific differential methylation proved to be absent from most imprinted genes, in EGC lines derived not only from 11.5, but also from 9.5dpc germ cells. This apparent conflict with the PGC data can be resolved if one assumes that the epigenetic changes involved in erasure of imprints continue after the cells are put into culture, reaching an imprint-free state once the EGC line is established. When these imprint-free EGCs have been used for making chimeras, some of the chimeras have shown growth retardation and skeletal defects (of ribs and spine). EGCs derived from 8.5dpc show heterogeneity of imprinted methylation patterns. Germline transmission, however, has been reported with imprint-free EGCs derived from 11.5dpc, as well as 8.5dpc PGCs. In human EGC lines, the epigenetic status of undifferentiated cells has not been examined, but after differentiation to fibroblast-like cells, three imprinted genes showed monoallelic expression (as in normal somatic tissues), and the fourth, *Igf2*, showed some relaxation of imprinting. These results suggest that there may be a considerable difference in the timing of imprint erasure between humans and mice. According to Baker (1963), there is also a difference between humans and mice in the timing of entry into meiosis: relative to colonization of the genital ridge, entry into meiosis is later and less well-synchronized in human than in mouse germ cells.

SUMMARY

The germ cell lineage in the mouse is not predetermined, but is established during gastrulation, in response to signaling molecules acting on a subset of epiblast cells that move through the primitive streak together with extra-embryonic mesoderm precursors. After migration to the site of the future gonads, germ cell sex determination is achieved, with germ cell phenotype in male and female embryos diverging. Germ cells that take the female pathway enter prophase of the first meiotic division five or six days after the birth of the germ cell lineage, whereas germ cells that take the male pathway exit the cell cycle and remain in G1/G0 until after

birth, when spermatogenesis begins. Site-specific DNA methylation of imprinted genes is erased in germ cells at about the time of entry into the future gonads, and new imprints are established later. In culture, germ cells respond to certain growth factors by proliferating indefinitely. These immortalized embryonic germ cell (EGC) lines are chromosomally stable and pluripotent, closely resembling the embryonic stem cell (ESC) lines derived from blastocyst-stage embryos. Human EGC lines have also been made.

KEY WORDS

Bisulfite sequencing A technique whereby the methylation of each CpG site in a defined stretch of DNA is determined.

Epigenetic Factors involved in influencing gene expression during development, without affecting DNA base sequence.

Gastrulation The period of embryonic development during which the definitive body plan is laid down. Cells from the primitive ectoderm pass through the "primitive streak" region to form a third layer, the mesoderm, between ectoderm and endoderm.

Meiosis The process by which germ cells (i.e., those in the ovaries or testes) divide to produce gametes. In meiosis I, homologous chromosomes exchange genetic material. In meiosis II, the two resulting diploid cells (i.e., which contain two sets of chromosomes) with their recombined chromosomes divide further to form two haploid gametes (i.e., which contain only one set of chromosomes).

Pluripotent Capable of giving rise to all the cell types in the fetus, but unable on its own to form a fetus.

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Chapter 15

Stem Cells in Extraembryonic Lineages

Tilo Kunath and Janet Rossant

INTRODUCTION

The establishment of trophoblast stem (TS) cell lines from mouse blastocysts and early trophoblast tissue has provided a model to study the trophoblast lineage in culture. TS cells can be expanded indefinitely, differentiate into trophoblast subtypes in culture, and contribute exclusively to the trophoblast lineage in chimeras. This chapter reviews development of the trophoblast lineage in the mouse, considers evidence for the existence and the location of trophoblast stem cell populations *in vivo*, and describes the work that led to the establishment of TS cell lines. Some applications of this cell culture system are also reviewed here. Finally, the extraembryonic endoderm lineage and a representative cell culture model are described briefly.

TROPHOBLAST LINEAGE

Trophoblast Development

An early priority of all mammalian embryos is to establish the extraembryonic lineages. The first such lineage to form is the trophoblast. In the mouse, this occurs by embryonic day (E) 3.5 when the pre-implantation morula embryo cavitates to form the blastocyst. The outer sphere of 40–50 trophectoderm (TE) cells of the early blastocyst contains the sole precursors of the entire trophoblast lineage. An eccentrically located clump of cells within the trophectodermal sphere is the inner cell mass (ICM). One day later, a second extraembryonic lineage, primitive endoderm (PrE) becomes apparent at the exposed surface of the ICM, now referred to as the epiblast. These three lineages are the foundations for development of the entire conceptus, and two of them (TE and PrE) are restricted to the extraembryonic compartment. The primitive ectoderm will also produce several extraembryonic tissues, such as the amnion, the allantois, and the mesodermal compartment of the definitive yolk sac (Figure 15-1).

The derivatives of the trophoblast lineage are essential for the survival of the embryo in the maternal uterine environment. It mediates implantation into the uterus and establishes a barrier for nutrient and waste exchange. It comprises a major portion of the placenta, where trophoblast cells take on endocrine and immunological roles in addition to the primary task of supplying the embryo with nutrient-rich blood and removing wastes. A role in embryo patterning is also established for this versatile lineage.

As mentioned previously, the trophoblast lineage is unambiguously present at blastocyst formation as the TE. There is a distinction between TE in contact with the ICM, or polar TE, and TE surrounding the blastocoel, or mural TE. The most distal or abembryonic cells in the mural TE are the first to differentiate into primary trophoblast giant cells, followed by cells laterally to the border of the ICM. The giant cells are aptly named, since they reach extraordinary sizes and possess very high DNA content. These cells undergo rounds of DNA synthesis (S phase) without intervening mitoses, in a process known as endoreduplication. The giant cells express several genes of the prolactin family, including placental lactogen I (*PL-I*) early in giant cell differentiation and later, they express *PL-II*.

Proliferating TE cells at the proximal region of the conceptus generate several trophoblast structures. At E6.5, trophoblast tissue in direct contact with the embryonic ectoderm forms the extraembryonic ectoderm (ExE). These cells are diploid and highly proliferative. Continuous with the ExE and immediately above it is the ectoplacental cone (EPC). The rate of proliferation is less in this tissue than in ExE, but the cells are still diploid. The EPC loses expression of several genes associated with ExE, such as *Fgfr2*, *Cdx2*, *Esrrb*, and Eomesodermin (*Eomes*), and it initiates expression of other genes, such as *Mash2* and *Tpbpa* (formerly *4311*). The outer periphery of the EPC has a lower mitotic index than the core, and these outer cells contribute secondary giant cells to the growing parietal yolk sac, as well as to the placenta itself (Figure 15-2). During

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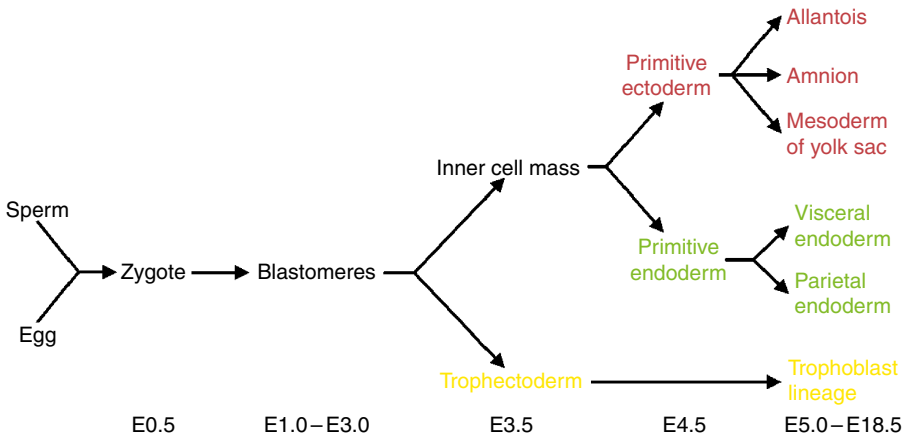


FIGURE 15-1 Extraembryonic lineages during mouse development. The primitive ectoderm, primitive endoderm, and trophoblast lineages are shown in red, green, and yellow, respectively. After fertilization, the zygote divides to produce blastomeres that eventually segregate into two lineages at blastocyst formation (E3.5): inner cell mass and trophectoderm. About one day later, the inner cell mass is further subdivided into the primitive ectoderm (or epiblast) and primitive endoderm. The primitive ectoderm will form all three germ layers of the embryo proper (not shown) and will contribute to several extraembryonic tissues: the allantois, the amnion, and the mesodermal part of the definitive (or visceral) yolk sac. The primitive endoderm differentiates into two major types of extraembryonic endoderm: visceral and parietal endoderm. The trophectoderm is the precursor to all trophoblast lineages in the placenta, as well as to the giant cell layer of the parietal yolk sac.

gastrulation, posterior mesoderm migrates into the extraembryonic region and becomes associated with trophoblast tissue of the ExE to form the chorion. This trophoblast tissue is now referred to as chorionic ectoderm (ChE) (Figure 15-2). At E8.5–9.0, the mesoderm-derived allantois fuses with the chorion, which further fuses with the base of the EPC, resulting in occlusion of the EPC cavity. These are the initial steps for forming a mature chorioallantoic placenta (Figure 15-2). In this mature tissue, the extraembryonic mesoderm and ChE combine to form the labyrinth, the site of nutrient and waste exchange with the maternal blood supply. The EPC differentiates into the spongiotrophoblast, a supporting tissue that is in intimate contact with the labyrinthine trophoblast. Peripheral giant cells are in direct contact with the maternal decidua, and are the invasive cells of the trophoblast. The maternal vasculature invades the spongiotrophoblast layer, and the endothelium is replaced with endovascular trophoblast as it enters the labyrinth.

Numerous genetic mutants have been characterized that affect various aspects of placental development, and they have been summarized in two reviews (Cross, 2000; Rossant and Cross, 2001). A few mutants of note are described here. The T-box gene, *Eomes*, is expressed in the TE of the blastocyst and the ExE of the early postimplantation embryo. A homozygous null mutation in this gene resulted in an embryonic lethal phenotype at the periimplantation stage. Blastocysts implanted into the uterus, but developed no further. The phenotype was caused by a cell-autonomous role for *Eomes* in trophoblast tissue as determined by chimeric analysis. The *Cdx2* mutation may have a similar defect to *Eomes*−/−, since the gene is also expressed in early trophoblast derivatives, and the knockout results in lethality at the time of implantation. Detailed analysis of *Cdx2*−/− embryos revealed a lack of mature TE, resulting in a failure of implantation (Strumpf *et al.*, 2005), a more severe phentotype than *Eomes*−/− embryos. However, the precise phenotype has not been reported yet. The orphan nuclear receptor, *Esrrb* (formerly known as *Errβ*), is expressed in the ExE and the ChE. Targeted deletion of this gene resulted in embryonic lethality at E9.5 with a failure of the chorion and an overproduction of giant cells. This gene is not required for the initial period of trophoblast proliferation, but it is required for its maintenance. The gene, *Tead4*, has recently been shown to have an essential and very early role in trophectoderm formation. This protein is not involved in polarization of blastomeres, but it is upstream of Cdx2, making it the earliest acting transcription factor in the trophoblast lineage (Yagi *et al.*, 2008). The lessons from these and other mutations point to the importance of lineage-specific transcription factors during trophoblast development.

Fgf Signaling and Trophoblast Progenitor Cells *In Vivo*

The polar TE at the embryonic pole of the blastocyst remains diploid and proliferative, and the mural TE differentiates into postmitotic giant cells. Sustained proliferation of polar TE cells is dependent on interactions with the ICM, and later with the epiblast. Transplanting an ectopic ICM to the mural TE region inhibits giant cell differentiation and induces a zone of proliferation. A model emerged whereby trophoblast proliferation was dependent on signals from the ICM, and in the absence of this stimulus the default pathway of giant cell differentiation would

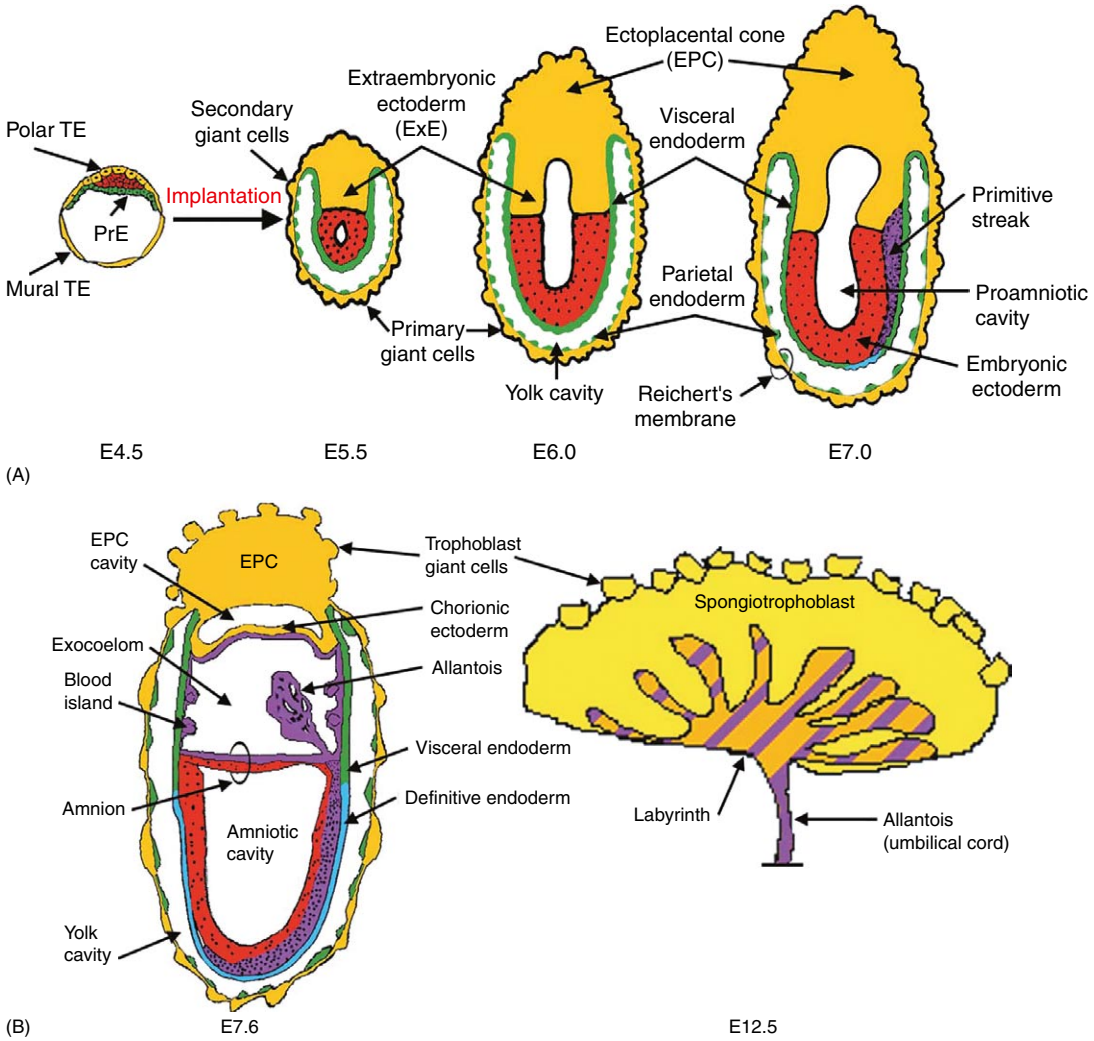


FIGURE 15-2 Post-implantation development. (A) Implantation to gastrulation. Based on their relative position to the inner cell mass, the trophectoderm (TE) cells of the E4.5 blastocyst have segregated into mural and polar TE. The polar TE grows into the blastocoel to produce the extraembryonic ectoderm and outward to form the ectoplacental cone (EPC). The mural TE differentiates into primary giant cells. Secondary giant cells line the outer surface of the EPC and contribute cells peripherally to the growing parietal yolk sac. At E6.5, the primitive streak forms at the posterior region of embryonic ectoderm adjacent to the extraembryonic ectoderm. By E7.0, definitive endoderm is beginning to emerge from the leading edge of the primitive streak. The primitive endoderm expands to line the entire surface of the former blastocoel, now known as the yolk cavity. The cells adjacent to the giant cell layer differentiate into parietal endoderm, and the cells in contact with the extraembryonic ectoderm and embryonic ectoderm become visceral endoderm. (B) Later development of extraembryonic lineages. By E7.5, posterior mesoderm has moved into the extraembryonic region and contributed to the chorion, amnion, and visceral yolk sac tissues. Three cavities are now present: the EPC cavity, the exocoelom, and the amniotic cavity. The definitive endoderm has replaced most of the visceral endoderm in the embryonic region after E7.5. The interaction of visceral endoderm and extraembryonic mesoderm induces blood islands in the definitive yolk sac region. The allantois, emerging from the posterior end of the embryo into the exocoelom, fuses with the chorion which also combines with the EPC to form the chorioallantoic placenta (E12.5). Chorionic ectoderm and mesoderm combined with allantoic mesoderm generate the labyrinth. The spongiotrophoblast is mostly derived from the EPC.

ensue. In agreement with this, the trophoblast-derived ExE and EPC differentiate into giant cells when explanted into culture. Contact with embryonic ectoderm, by placing the ExE or EPC into the amniotic cavities of E7.5 embryos ("embryonic pocket" culture), inhibited giant cell differentiation of the ExE only. Thus, the trophoblast tissue nearest the epiblast, the ExE, can respond to proliferation signals from the embryo, but the more distal EPC is refractory to them. This is the first indication of where the trophoblast stem cells may reside *in vivo*.

Clues to the identity of the embryo-derived signal came from expression and genetic studies of the fibroblast growth factor (FGF) signaling pathway. Embryos mutant for the *Fgf4* gene die shortly after implantation. Since this ligand is expressed in the ICM, it could have an autocrine function in maintaining ICM cells, a paracrine role in the proliferation of polar TE cells, or both. The reciprocal expression pattern of *Fgfr2* in the TE and the ExE lent support for the paracrine model. Two different targeted deletions of the *Fgfr2* gene resulted in different phenotypes on the same

genetic background. The deletion of exon 9, the alternatively spliced IIIc exon, resulted in a peri-implantation lethal phenotype similar to the *Fgf4* mutation. Surprisingly, deletion of the complete Ig III loop (exons 7, 8, and 9) resulted in a less severe phenotype, with lethality occurring at E10.5 because of a failure of the placental labyrinth. The IIIc deletion could be a dominant–negative mutation, since there is a possibility that a soluble FGFR2 IIIb variant is produced. However, heterozygous mice were normal and fertile.

On the other hand, the complete Ig III deletion might be a hypomorphic allele. They show by Western analysis that a truncated protein with the remaining two Ig loops and a complete intracellular domain is produced. Although it cannot bind FGF ligands, it may participate in some ligand-independent signaling. Regardless of which *Fgfr2* allele is the complete loss-of-function mutation, an essential role for FGF signaling in trophoblast development is unmistakable. An *Fgfr2 IIIb* isoform-specific knockout resulted in a phenotype that did not have trophoblast defects as reported for the two *Fgfr2*-null alleles. The mice died at birth with severe lung and limb abnormalities. In addition, the placental rescue of an *Fgfr2*-null allele by tetraploid aggregation resulted in a phenocopy of the *IIIb*-specific mutation. This implicates the IIIc isoform of FGFR2 as the trophoblast-specific receptor and predicts that an *IIIc*-specific mutation should show trophoblast defects similar to one of the null mutations. The *Grb2* and *FRS2*α mutations may also have defects in the initial period of proliferation of the polar TE by disabling the FGF signal transduction pathway. Grb2 is an adaptor for several signal pathways, but FRS2 α is specific to FGF signaling.

Based on gene expression studies, FGF-dependent trophoblast stem cells are likely present from E3.5 to E8.5 (for five days). *Fgfr2* expression is down-regulated in the ChE by E8.5, and *Eomes*, an essential gene for the early trophoblast, is also repressed. A more precise prediction of when and where early trophoblast progenitors reside came from studies on the distribution of activated diphosphorylated mitogen-activated protein kinases, Erk1 and Erk2 (dpMAPK) during early development. These studies revealed regions of the embryo where MAPK was activated, a common target in several signal transduction pathways. The early ExE (E5.5) was positive for dpMAPK, but most of the epiblast and visceral endoderm (VE) were not. The region of ExE positivity become more restricted to the trophoblast cells closest to the epiblast as development continued. This ring of dpMAPK staining persisted in the ChE after gastrulation occurred and contact with the epiblast was lost. The MAPK activation in ExE and ChE was attributed to FGF signaling, since a specific inhibitor (SU5402) abolished staining in this region and not others (Corson *et al.*, 2003).

As will be detailed later in this chapter, FGF-dependent TS cell lines can be derived from blastocysts and early post-implantation trophoblast tissue. Studies by Uy *et al.* (2002) used TS cell line derivations as an assay to determine which tissues could generate characteristic TS cell cultures. Although this may not precisely determine where the multipotent progenitors exist *in vivo*, it should definitively exclude regions and embryological times in which they do *not* exist. The ability to derive TS cell lines was demonstrated for embryos from the early blastocyst stage to as late as the 10-somite pair stage (~E8.0). From post-implantation embryos, TS cell lines could only be derived from ExE or ChE, and not from the EPC or embryonic tissues; they could not be derived from trophoblast tissue at later stages. It was also noted that TS cell lines could be derived from all regions of the ExE, including the cells adjacent to the EPC (farthest from the epiblast). Later in development, the ChE, a trophoblast tissue not in direct contact with the epiblast, efficiently produced TS cell lines. This suggested that the inductive FGF signal supplied by the embryo at this stage is diffusible over a long range, or that it is produced from other tissues, such as the extraembryonic mesoderm or endoderm. The areas of dpMAPK positivity and the regions where TS cell lines could be derived did not perfectly coincide. TS cell cultures could be obtained from ExE cells distant from the epiblast and regions of the chorion that were not positive for dpMAPK. However, *Fgfr2* expression did persist in these dpMAPK-negative regions, suggesting that cells could respond to an FGF signal, but were not doing so *in vivo*. For this reason, trophoblast cells in ExE–ChE that are positive for dpMAPK may more accurately represent the trophoblast stem cell population *in vivo*.

The current model proposes that FGF4 produced by the ICM is necessary for the proliferative maintenance of the polar TE, and that the lack of FGF signaling in the mural region results in giant cell differentiation. After implantation, the epiblast continues to produce FGF4 that signals to the overlying ExE, resulting in MAPK activation and maintenance of the trophoblast progenitor population. The FGFR2 IIIc isoform is predicted to be the trophoblast-specific FGF receptor *in vivo*. Essential transcription factors for the maintenance of this population include *Cdx2*, *Eomes*, and *Esrrb*. After gastrulation and the formation of the chorion, portions of the ChE remain positive for dpMAPK, but the source and identity of the FGF signal is unclear at this point (Figure 15-3).

The FGF-dependent progenitors are not present after chorioallantoic fusion, but the placenta is still required to undergo a substantial increase in size and is likely under the control of different signaling pathways. The TGFβ signaling pathway activated by Nodal is a possible candidate for this signal. An insertional mutation at the *nodal* locus resulted in a placental defect with excessive giant cell formation and a lack of spongiotrophoblast and labyrinth development. Unlike *Fgfr2* or the transcription factors *Cdx2*, *Eomes*, and *Esrrb*, *nodal* is not expressed in the early ExE or ChE. Its expression in the placenta is specific to the spongiotrophoblast layer (an

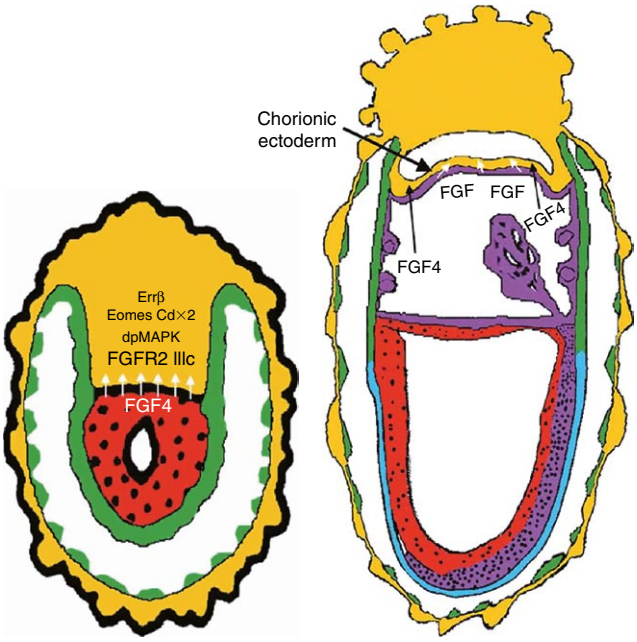


FIGURE 15-3 Model of early trophoblast development. (A) During early post-implantation development, FGF4 produced by the epiblast signals to the overlying extraembryonic ectoderm (white arrows) using the FGFR2 IIIc isoform. This leads to activation of the MAPK pathway, as indicated by diphosphorylated MAPK (dpMAPK). Downstream transcription factors essential for the maintenance of this lineage include Eomes, Cdx2, and Errβ (Esrrb). (B) After gastrulation, the chorionic ectoderm is distant from the embryo proper, but maintains dpMAPK activity. FGF4 may be diffusing over a long range, or a different FGF may be supplied by the underlying extraembryonic mesoderm.

EPC descendant), begins at E9.5, and continues until term. Interestingly, a compound null–hypomorph *nodal* mutation resulted in a less severe placental defect in which the giant cell and spongiotrophoblast layers were expanded at the expense of the labyrinth. This suggests that Nodal-dependent progenitors may be residing in the labyrinth and that the source of the proliferative signal has shifted from the embryo to the spongiotrophoblast.

TROPHOBLAST STEM CELL LINES

Derivation of Trophoblast Stem Cell Lines

The culture of ExE or EPC tissue in most conditions resulted in giant cell differentiation. This led to the proposal that the default state of early trophoblast tissue is giant cells. The accumulating evidence that FGF signaling was important for trophoblast development and could prevent giant cell transformation led Dr Satoshi Tanaka to revisit ExE explant cultures. FGF4 reduced not only giant cell formation in ExE explants, but also the amount of EPC-like differentiation. Encouraged by these results, ExE from E6.5 embryos was disaggregated into single cells and plated on embryonic fibroblasts (EMFIs) in the presence of FGF4 and its essential cofactor heparin. This resulted in the formation of flat epithelial colonies that could be passaged indefinitely. Identical cell lines could also be derived from E3.5 blastocyst outgrowths (Tanaka *et al.*, 1998).

TS cell lines expressed *Fgfr2* and the transcription factors characteristic of trophoblast progenitors *in vivo*: *Cdx2*, *Eomes*, and *Esrrb*. Removal of FGF4, heparin, or the EMFIs resulted in giant cell differentiation (Figure 15-4A, B) and complete down-regulation of these four markers. During the differentiation process, markers of intermediate trophoblast tissue, such as *Mash2* and *Tpbpa*, were expressed. The cell culture-derived giant cells exhibited increased ploidy and expressed the giant cell marker, *Pl-I*, like their *in vivo* counterparts. TS cells did not express the ES cell or early epiblast marker *Oct4*, the mesoderm marker *Brachyury*, or the endoderm marker *Hnf4*. FACS analysis and visual inspection of the cultures revealed that TS cell cultures are heterogeneous even when grown in stem cell conditions (Figure 15-4C, D). TS cell cultures could also be maintained in EMFI-conditioned medium, suggesting that EMFIs produce a soluble factor or factors important for TS cell self-renewal.

The developmental potential of TS cells was tested in chimeras generated by blastocyst injections. Green fluorescent protein (GFP)-transgenic TS cells were analyzed in chimeras from E6.5 to term (E18.5). All chimeras had TS cell contributions exclusively in trophoblast tissues (Figure 15-4E, F). There were no examples of contributions to the embryo proper or to the visceral yolk sac and amnion, two extraembryonic membranes that do not contain trophoblast cells. In addition, all subtypes of the trophoblast lineage could be colonized. In early embryos, GFP-TS cells were in the ExE, EPC, and giant cells. In older conceptuses, the labyrinth and spongiotrophoblast had GFP-TS cell contributions. The exclusive, tissue-specific restriction observed in chimeras indicates that TS cells are committed to the trophoblast lineage and retain the potential to differentiate into its many cell types.

Although TS cells can produce some remarkable chimeras with high contributions to the trophoblast lineage, improvements in its efficiency are required before this can be used as a routine assay to test the potential of manipulated TS cell lines. The percentage of observing chimeras from the most efficient method (blastocyst injection) is 45% of embryos recovered. However, the frequency of obtaining *high-contribution* chimeras is between 5% and 10%. There may be several reasons for this low efficiency. First, the heterogeneity of the TS cell cultures suggests that only a fraction of the cells may be true stem cells. Any method to identify cells with greater stem cell potential and separate them from differentiated cells should improve the efficiency and extent of contribution during chimera production. Diploid cells can be separated from higher ploidy cells by fluorescence-activated cell sorting (FACS) with Hoechst 33342 dye, and a reduction in heterogeneity is observed by morphology and FACS analysis. However, this procedure only excludes one of the trophoblast subtypes: polyploid giant cells. A more

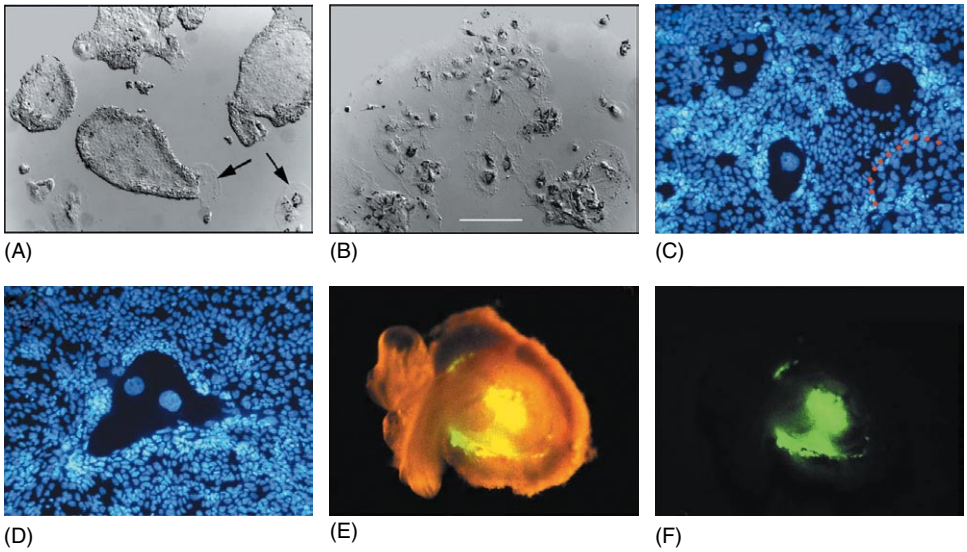


FIGURE 15-4 TS cells and a TS cell chimera. TS cells grown in: (A) stem cell; and (B) differentiative conditions. (A) Differential interference contrast (DIC) micrograph of several TS cell colonies grown in stem cell conditions. They form tight epithelial sheets with occasional differentiated giant cells (arrows) at their periphery. (B) DIC micrograph of six-day differentiated TS cells showing a drastic change in cell morphology to the characteristic giant cells. The bar is 50 μm. (C, D) Two examples of confluent stem cell cultures of TS cells stained with Hoechst 33342. The huge nuclei and vast cytoplasm of the giant cells are evident. There are also cells with intermediate sized nuclei (dots in panel C, for example). (E, F) An E8.5 TS cell chimera generated by blastocyst injection of green fluorescent protein–TS cells. The embryo with placenta was observed under UV fluorescence with (E) partial bright-field and (F) dark-field optics.

precise method of isolation—for example, FACS for a specific cell-surface marker such as FGFR2 or high-efflux properties such as the side population—should further narrow the stem cell population. To date, attempts to rescue a genetic trophoblast defect by TS cell chimerism have not been reported, but identifying and isolating TS cells with maximum developmental potential should help in this area.

Trophoblast Stem Cells as Models of Trophoblasts

The TS cell culture system was used to investigate the role of the orphan nuclear receptor, ERRβ, through the use of a small molecule inhibitor. Diethylstilbestrol (DES) is a synthetic estrogen that is a potent agonist for the classical estrogen receptors, ERα and ERβ. It was demonstrated that DES has the opposite effect on the three ER-related receptors: ERRα, ERRβ, and ERRγ. ERRs are constitutively active transcription factors and do not require a ligand for activation. Since TS cells and ExE express high levels of *Esrrb* (*Err*β, the effects of DES treatment were investigated. DES specifically induced TS cell differentiation toward a trophoblast giant cell phenotype, and estradiol had no effect. To investigate the effect of DES treatment on trophoblast tissue *in vivo*, pregnant mice were fed DES during early placentation (E4.5 to 8.5), and their placentas were examined at E9.5. Strikingly, the DES-treated placentas did not have the usual trilayered placenta consisting of spongiotrophoblast, labyrinth, and giant cells. Instead, they consisted of multiple layers of giant cells similar to *Esrr*-mutant placentas. This strongly suggested that the effects of DES in the placenta and in TS cells are mediated through only one of the ERRs: ERRβ. This illustrates that ERRβ is essential for the maintenance of TS cells in culture, and the trophoblast progenitor population *in vivo*.

Since the first report of TS cell lines, several laboratories have used this stem cell culture system for a diverse range of studies. A very interesting result was obtained when *Oct4* was conditionally repressed in ES cells. The *Oct4*-repressed cells transformed into trophoblast giant cells at the expense of embryonic lineages that ES cells normally produce under differentiative conditions. Even more striking was the establishment of TS cell cultures when FGF4 was added to ES cells during the down-regulation of *Oct4* (Niwa *et al.*, 2000). This implicated Oct4 as a major repressor of the trophoblast lineage. Avilion *et al.* (2003) used the derivation of TS cell lines as an assay to determine the necessity of the high-mobility group box transcription factor, *Sox2*, for this lineage. They determined that *Sox2* is an essential gene for the trophoblast lineage, since null TS cells could not be derived. Adelman *et al.* (2000) derived TS cells mutant for the hypoxia-responsive transcription factor *Arnt*. They went on to show that *Arnt* −/− TS cells were deficient in forming the intermediate EPC-like trophoblast cells during differentiation in culture. Yan *et al.* (2001) used TS cells to study the effects of retinoic acid (RA) treatment in culture. They also found that TS cells skipped the intermediate, *Mash2*+ trophoblast subtype and differentiated directly into giant cells when treated with RA. Mak *et al.* (2000) found that transfection of TS cells

with *nodal* decreases giant cell formation, and that JunB activity increases it. Female TS cells were used by Mak *et al.* to show that paternally imprinted X-chromosome inactivation observed in the female trophoblast lineage *in vivo* is maintained in TS cells. Furthermore, the imprinting is maintained by stable association of the Polycomb proteins, Eed and Enx1, with the inactive X-chromosome. The role of α7 integrin in trophoblast attachment to different extracellular substrates was investigated with the TS cell culture system. Suppressor of cytokine signaling 3 (*SOCS3*)-mutant TS cells were derived and shown to have an increased tendency to form giant cells, at the expense of EPC-like cells, similar to the phenotype observed for the *SOC3* −/− placenta *in vivo*. A targeted mutation against the mitochondrial transmembrane guanosine triphosphatase, mitofusin 2 (*Mfn2*), resulted in a giant cell defect and lethality by E11.5. *Mfn2* −/− TS cell lines were derived and shown to have severe morphological defects in their mitochondria. They were spherical and small, instead of the long mitochondrial tubules observed in wild-type TS cells. These various reports illustrate that TS cell lines provide a useful model to study the trophoblast lineage in the mouse.

EXTRAEMBRYONIC ENDODERM LINEAGE

Extraembryonic Endoderm Development

The primitive endoderm (PrE) lineage first becomes apparent on the blastocoelic surface of the ICM at E4.5. The developmental path taken by the PrE depends on the tissue with which it interacts. The PrE in contact with the extraembryonic and embryonic ectoderm differentiates into VE, and the PrE in contact with the trophoblast giant cell layer differentiates into parietal endoderm (PE) (Figure 15-2). The PE collaborates with the giant cell layer to form Reichert's membrane. The combination of the PE, giant cells, and a thick basement membrane is the parietal yolk sac. The VE is a complex lineage with roles in nutrient delivery, embryonic cavitation, anterior induction, and hemangioblast induction (Bielinska *et al.*, 1999).

Once the initial PrE cells are established, the GATA factors, GATA4 and GATA6, are major players in the maintenance and elaboration of this lineage. *GATA4*-mutant embryos form VE, but express elevated levels of *GATA6*, suggestive of some functional redundancy. The embryos die around E9.5 with heart defects. The *GATA6* mutation is more severe, with serious VE defects and lethality occurring between E5.5 and 7.5. An instructive role for these proteins in extraembryonic endoderm specification was shown by ectopic expression in ES cells.

The transition of VE to PE first occurs at the periphery of the ICM as the cells migrate onto the inner surface of the mural TE at the blastocyst stage. The VE grows as an epithelial layer with coherent growth, and the PE cells grow individually and scatter on the giant layer as they lay down matrix proteins. The differentiation of VE to PE is considered by some to be the first epithelial-to-mesenchymal transition in development. Studies in embryonal carcinoma cells identified cyclic adenosine monophosphate as an inducer of PE, if the cells were first differentiated into VE by RA treatment. The ligand, parathyroid hormone-related protein (PTHrP), was later suggested to be an endogenous inducer of PE. PTHrP is produced by the giant cell layer, and PE cells express its receptor, the G-protein-coupled receptor, type I PTHrP-R, at high levels. The zinc-finger transcription factor, Snail, was identified as an immediate early target of PTHrP signaling in ES cells, and was shown to be expressed in PE *in vivo*. *Snail* and the highly related gene, *Slug*, are important for epithelial–mesenchymal transitions in several species. In *Snail*−/− embryos, the initial mesoderm is formed, but it remains epithelial-like in agreement with the defined role of *Snail* in epithelial-mesenchymal transitions. *Snail*−/ − embryos are smaller than wild-type littermates at E7.5 and die before E8.5. A phenotype in the VE-to-PE transition was not reported on, but such a defect would not be inconsistent with the time of lethality.

Extraembryonic Endoderm Progenitors?

PrE is considered to be the progenitor of both VE and PE, but this primitive cell type only persists one day after implantation (E5.5). After this point, both extraembryonic endoderm layers (VE and PE) continue to grow extensively. Chimera studies have shown that PrE cells have the potential to contribute to both VE and PE in a single chimera. Observations, such as the sequential appearance of VE cells followed by PE cells during the differentiation of ES and EC cells, have biased opinions on how these cell types arise *in vivo*. These observations led to a model in which VE cells continually produce more PE cells in the marginal zone (the proximal region of yolk cavity). However, these experiments do not conclusively prove such a relationship *in vivo*. It is possible that the VE and PE layers have separate and self-sufficient cell populations after an early point in development (e.g., by E6.5). One possibility is that PE progenitors exist at the distal tip of the parietal yolk sac, and that they contribute descendants that migrate up towards the marginal zone. The VE layer may have a similar progenitor zone to maintain its population. Mitotic indices in both tissues measured at E7.5 were similar. An alternative theory puts extraembryonic endoderm stem cells in the marginal zone between the VE and the PE. Although this region has been proposed to be the site of VE-to-PE transition, direct lineage analysis of these cells has not been performed. Real-time imaging of embryo–embryo chimeras (harboring different fluorescent transgenes) should answer some of these questions. The recent derivation of extraembryonic endoderm (XEN) cell lines from mouse blastocysts has provided a cell culture model to address

some of the issues discussed above (Kunath *et al.*, 2005). XEN cells can be cultured indefinitely, express markers of the extraembryonic endoderm lineage, and exclusively contribute to this lineage in chimeras.

SUMMARY

Gene expression studies (e.g., *Eomes*), TS cell line derivation potential, and MAPK activity in the embryo have led to a precise prediction of when and where trophoblast multipotent progenitors exist *in vivo*. The ExE cells close to the epiblast and a discrete population of ChE are the most likely locations for these early progenitors. However, by E8.5, the FGF-dependent progenitors do not exist and may be replaced by a different class of multipotent cells. The extraembryonic VE and PE layers exhibit substantial growth during development, but a defined progenitor or stem cell population has yet to be identified in this cell lineage.

KEY WORDS

Blastocyst A mammalian embryo, just prior to implantation, consisting of a hollow ball of cells surrounding a smaller clump of cells known as the inner cell mass. The blastocyst is the source material to derive embryonic stem (ES), trophoblast stem (TS), and extraembryonic endoderm (XEN) cell lines.

Primitive endoderm An extraembryonic lineage that forms part of the yolk sac. The primitive endoderm does not contribute to endodermal tissues, such as the gut, liver, or pancreas. These are derived from definitive endoderm, a distinct lineage from primitive endoderm.

Trophectoderm The outer layer of cells of the blastocyst. Trophectoderm cells are the sole precursors to the trophoblast lineage of the placenta, and they do not contribute cells to the embryo proper.

Trophoblast A general term used to describe all the cell types of the developing and mature placenta derived from the trophectoderm. In the mouse, the trophoblast lineage would include extraembryonic ectoderm, ectoplacental cone, trophoblast giant cells, chorionic ectoderm, labyrinthine trophoblast, and spongiotrophoblast.

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Chapter 16

Amniotic Fluid-Derived Pluripotential Cells

Anthony Atala

INTRODUCTION

For over 70 years, human amniotic fluid has been used in prenatal diagnosis, and its use has proven to be safe, reliable, and relatively simple. By screening this fluid and the cells contained within it, a variety of genetic and developmental disorders of the fetus can be diagnosed. However, recent studies involving the cells of the amniotic fluid suggest that this fluid may be much more than a simple diagnostic tool—it may, in fact, contain a powerful new therapy that could one day be used to treat and cure a multitude of disorders. These studies have shown that amniotic fluid contains a novel type of stem cell which is capable of being maintained in an undifferentiated state in culture for long periods, and can be induced to differentiate into many different cell types. Because these amniotic fluid stem cells (AFSCs) can be obtained without the destruction of an embryo, their use can sidestep the myriad of ethical, religious, and political issues that surround the use of embryonic stem cells. Thus, it is possible that we will soon see the development of AFSC-based therapies for newborns with congenital malformations, as well as a number of adult disorders and injuries.

In this chapter, we will describe the experiments that led to the isolation and characterization of AFSCs. We will also describe the various lineages that these cells have been differentiated into thus far, and discuss the future of these promising new stem cells.

AMNIOTIC FLUID AND AMNIOCENTESIS

The first reported amniocentesis took place in 1930, when attempts were being made to correlate the cytological examination of cell concentration, count, and phenotypes in the amniotic fluid to the sex and health of the baby. Since then the development of techniques of karyotype, the discovery of reliable diagnostic markers, such as α-fetoprotein, and the development of ultrasound-guided amniocentesis, have greatly increased the reliability of the procedure as a valid diagnostic tool, as well as improving the safety of the procedure.

Amniocentesis provides a safe method of isolating cells from the fetus, which can then be karyotyped and examined for chromosomal abnormalities. In general, the protocol consists of acquiring 10 to 20 ml of fluid using a transabdominal approach. Amniotic fluid samples are then centrifuged, and the cell supernatant is resuspended in culture medium. Approximately 10^4 cells are seeded on 22 × 22 mm cover slips. Cultures are grown to confluence for three to four weeks in 5% CO_2 at 37ºC, and the chromosomes are characterized from mitotic phase cells.

Amniocentesis is performed typically around 16 weeks of gestation, although in some cases it may be performed as early as 14 weeks when the amnion fuses with the chorion, and the risk of bursting the amniotic sac by needle puncture is minimized. Amniocentesis can be performed as late as term. The amniotic sac is usually noticed first by ultrasound around the 10-week gestational time point.

Amniotic fluid cell culture consists of a heterogeneous cell population displaying a range of morphologies and behaviors. Studies on these cells have characterized them into many shapes and sizes, varying from 6 to 50 μm in diameter, and from round to squamous in shape. Most cells in the fluid are terminally differentiated along epithelial lineages, and have limited proliferative and differentiation capabilities. Previous studies by Cremer *et al.* (1981) have noted an interesting composition of the fluid consisting of a heterogeneous cell population expressing markers from all three germ layers.

Much research has been conducted on the source of these cells, and on the fluid itself. Current theories suggest that the fluid is largely derived from urine and peritoneal fluid from the fetus, as well as from some ultrafiltrate from the plasma of the mother entering though the placenta. The cells in the fluid have been shown to be overwhelmingly from the fetus, and are thought to be mostly cells sloughed off the epithelium, digestive, and urinary tract of the fetus, as well as off the amnion.

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Our laboratory investigated the possibility of isolating a progenitor cell population from amniotic fluid. The amniotic fluid was from normal fetuses, obtained using a transabdominal approach from 14 to 21 weeks of gestation. Initially, male fetuses were used to preclude the possibility of maternal-derived cells.

ISOLATION AND CHARACTERIZATION OF PROGENITOR CELLS

A pluripotential sub-population of progenitor cells in the amniotic fluid can be isolated through positive selection for cells expressing the membrane receptor c-kit, which binds to the ligand stem cell factor. Roughly 0.8% to 1.4% of cells in amniotic fluid have been shown to be c-kit(pos) in analysis by fluorescence-activated cell sorting.

The progenitor cells maintain a round shape for one week after isolation when cultured in nontreated culture dishes. In this state, they demonstrate a very low proliferative capability. After the first week, the cells begin to adhere to the plate and change their morphology, becoming more elongated and proliferating more rapidly to reach 80% confluence with a need for passage every 48 to 72 hours. No feeder layers are required for either maintenance or expansion. The progenitor cells derived from amniotic fluid show a high self-renewal capacity with >300 population doublings, far exceeding Hayflick's limit. The doubling time of the undifferentiated cells is noted to be 36 hours, with little variation with passages.

These cells have been shown to maintain a normal karyotype at late passages, and have normal G1 and G2 cell cycle checkpoints. They demonstrate telomere length conservation in the undifferentiated state, as well as telomerase activity even in late passages as shown by Bryan *et al.* (1998). Analysis of surface markers shows that progenitor cells from amniotic fluid expressed human embryonic stage-specific marker SSEA4 and the stem cell marker OCT4, but they did not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, or TRA1-81, to name a few. This expression profile is of interest as it demonstrates expression by the amniotic fluid-derived progenitor cells of some key markers of embryonic stem cell phenotype, but not the full complement of markers expressed by embryonic stem cells. This hints that the amniotic cells are not as primitive as embryonic cells, and yet maintain greater potential than most adult stem cells. Work by Thomson *et al.* (1998) shows that, although the amniotic fluid progenitor cells form embryoid bodies *in vitro* that stain positive for markers of all three germ layers, these cells do not form teratomas *in vivo* when implanted into immunodeficient mice. Lastly, cells expanded from a single cell maintain similar properties in growth and potential as the original mixed population of the progenitor cells.

DIFFERENTIATION POTENTIAL OF AMNIOTIC PROGENITOR CELLS

The progenitor cells derived from human amniotic fluid are pluripotent, and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, and hepatic phenotypes *in vitro*. Each differentiation has been performed through proof of phenotypic (Figure 16-1) and biochemical (Figure 16-2) changes consistent with the differentiated tissue type. We will describe each set of differentiations separately.

Adipocytes

To promote adipogenic differentiation, the progenitor cells can be induced in dexamethasone, 3-isobutyl-1-methylxanthine, insulin, and indomethacin. The progenitor cells cultured with adipogenic supplements change their morphology from elongated to round within eight days. This coincides with the accumulation of intracellular droplets. After 16 days in culture, more than 95% of the cells have their cytoplasm filled with lipid-rich vacuoles.

Adipogenic differentiation also demonstrates the expression of an adipogenic-specific transcription factor, and of lipoprotein lipase, as noted by Kim *et al.* (1998) and Rosen *et al.* (1999). Expression of these genes is noted in the progenitor cells under adipogenic conditions, but not in undifferentiated cells (Figure 16-2B).

Endothelial Cells

The amniotic fluid progenitor cells can be induced to form endothelial cells by culture in endothelial basal medium on

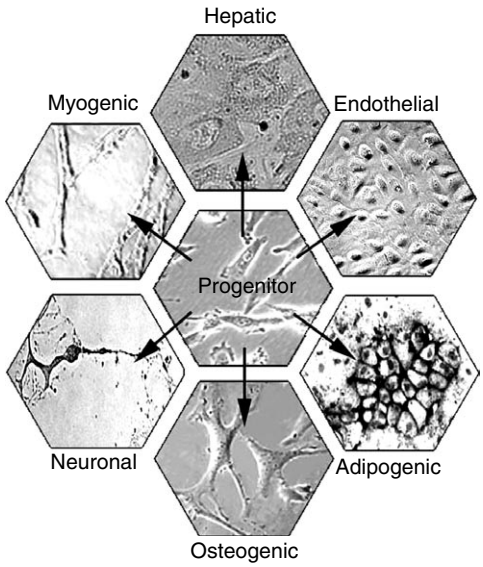


FIGURE 16-1 The isolated progenitor cells were capable of differentiation into multiple cell types, including muscle, liver, endothelial cells, adipocytes, osteoblasts, and neurons.

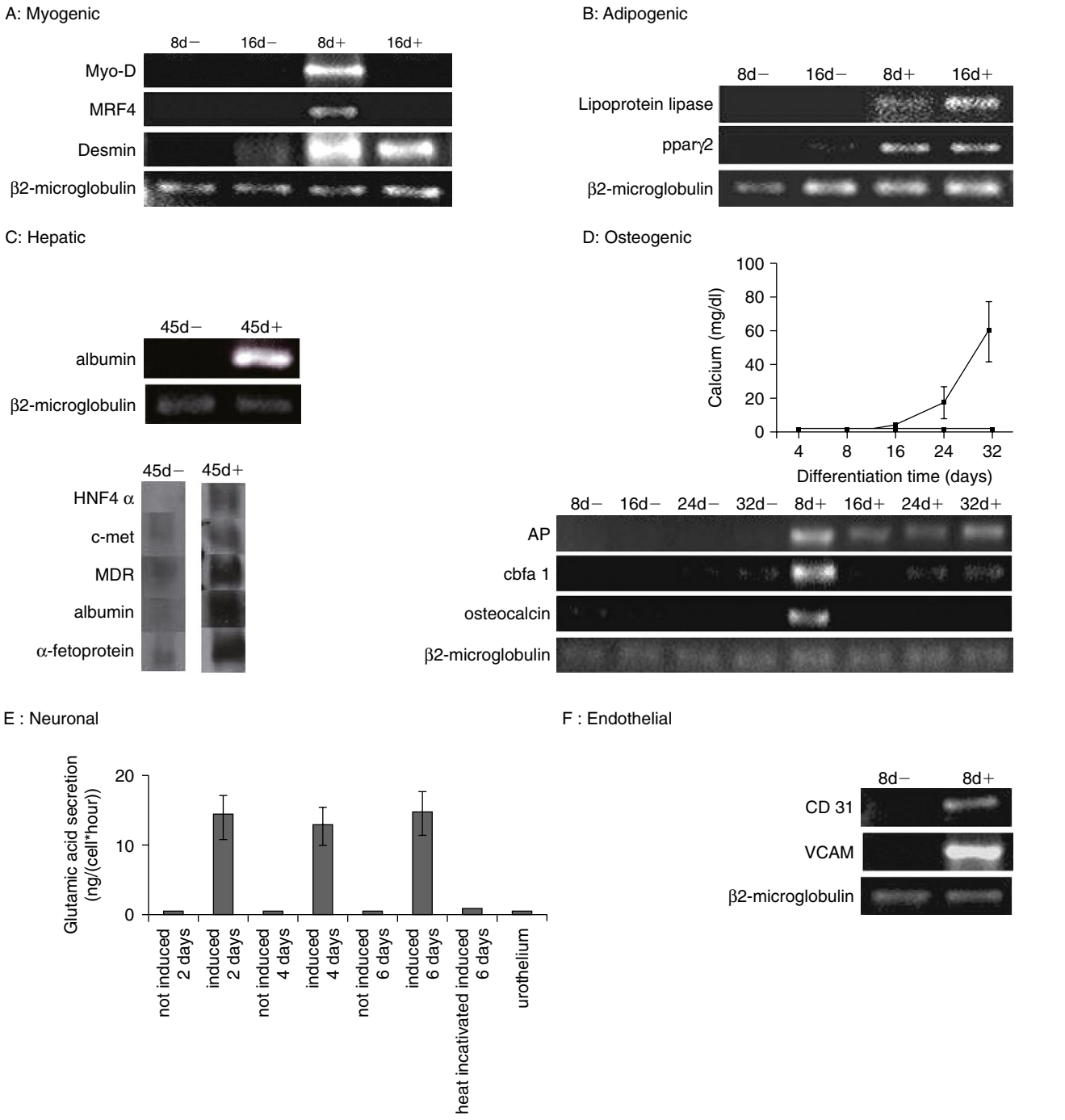


FIGURE 16-2 The differentiated cell types expressed functional and biochemical characteristics of the target tissue. (A) Myogenic-induced cells showed a strong expression of *desmin* expression at day 16 (lane 4). *MyoD* and *MRF4* were induced with myogenic treatment at day 8 (lane 3). Specific PCR-amplified DNA fragments of *MyoD*, *MRF4*, and *desmin* could not be detected in the control cells at days 8 and 16 (lanes 1 and 2). (B) Gene expression of *pparγ2* and *lipoprotein lipase* in cells grown in adipogenic-inducing medium was noted at days 8 and 16 (lanes 3 and 4). (C) RT-PCR revealed an up-regulation of *albumin* gene expression. Western blot analyses of cell lysate showed the presence of the hepatic lineage-related proteins HNF-4α, c-met, MDR, albumin, and α-fetoprotein. Undifferentiated cells were used as negative control. (D) Osteogenic-induced progenitor cells showed a significant increase of calcium deposition starting at day 16 (solid line). No calcium deposition was detected in progenitor cells grown in control medium or the negative control cells grown in osteogenic conditions (dashed line). RT-PCR showed presence of *cbfa1* and *osteocalcin* at day 8 and confirmed the expression of *AP* in the osteogenic-induced cells. (E) Only the progenitor cells cultured under neurogenic conditions showed the secretion of glutamic acid in the collected medium. The secretion of glutamic acid could be induced (20 minutes in 50-mM KCl buffer). (F) RT-PCR of progenitor cells induced in endothelial medium (lane 2) showed the expression of *CD31* and *VCAM*.

gelatin-coated dishes. Full differentiation is affected with one month in culture; however, phenotypic changes are noticed within one week of initiation of the protocol. Human-specific endothelial cell surface marker (P1H12), factor VIII (FVIII), and kinase insert domain-containing receptor are specific for differentiated endothelial cells. The differentiated cells stain positively for FVIII, KDR, and P1H12. The amniotic fluid-derived progenitor cells do not stain for endothelial-specific markers and, once differentiated, are able to grow in culture and form capillary-like structures *in vitro*. These cells also express molecules that are not detected in the progenitor cells on RT-PCR analysis (Figure 16-2F).

Hepatocytes

For hepatic differentiation, the progenitor cells are seeded on Matrigel- or collagen-coated dishes at different stages and cultured in the presence of hepatocyte growth factor, insulin, oncostatin M, dexamethasone, fibroblast growth factor 4, and monothioglycerol for 45 days. After seven days of the differentiation process, cells exhibit morphological changes from an elongated to a cobblestone appearance. The cells show positive staining for albumin at day 45 after differentiation and also express the transcription factor HNF4α, the c-met receptor, the MDR membrane transporter, albumin, and α-fetoprotein (Figure 16-2C).

Myocytes

Myogenic differentiation is induced in the amniotic fluid-derived progenitor cells, by culture in media containing horse serum and chick embryo extract on a thin gel coat of Matrigel. To initiate differentiation, the presence of 5-azacytidine in the media for 24 hours is necessary. Phenotypically, the cells can be noted to organize themselves into bundles that fuse to form multinucleated cells. These cells express sarcomeric tropomyosin and desmin, both of which are not expressed in the original progenitor population.

Interestingly, the development profile of cells differentiating into myogenic lineages mirrors a characteristic pattern of gene expression seen with embryonic muscle development, as shown in work by Rohwedel *et al.* (1994) and Bailey *et al.* (2001) (Figure 16-2A).

Neuronal Cells

For neurogenic induction, the amniotic progenitor cells are induced in dimethyl sulfoxide, butylated hydroxyanisole, and neuronal growth factor. Progenitor cells cultured in neurogenic conditions change their morphology within the first 24 hours. Two cell populations are apparent: morphologically large, flat cells and small, bipolar cells. The bipolar cell cytoplasm retracts towards the nucleus, forming contracted multipolar structures. Over the subsequent hours, the cells display primary and secondary branches and cone-like terminal expansions. The induced progenitor cells show a characteristic sequence of expression of neural-specific proteins, including those expressing neuroepithelial, neuron, and glial differentiation (Figure 16-2E).

Osteocytes

Osteogenic differentiation was induced in the progenitor cells with use of dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate. The progenitor cells maintained in this medium demonstrated phenotypic changes within four days, with a loss of spindle-shape phenotype and development of an osteoblast-like appearance with finger-like excavations into the cytoplasm. At 16 days, the cells aggregated, showing typical lamellar bone-like structures. In terms of functionality, these differentiated cells demonstrate a major feature of osteoblasts, which is to precipitate calcium. Differentiated osteoblasts from the progenitor cells are able to produce alkaline phosphatase (AP), and to deposit calcium consistent with bone differentiation. The undifferentiated progenitor cells lacked this ability.

The progenitor cells in osteogenic medium express specific genes implicated in mammalian bone development, in a pattern consistent with the physiological analog (Figure 16-2D).

Renal Cells

End stage kidney disease has reached epidemic proportions in the United States. Currently, dialysis and allogenic renal transplant remain the only treatments for this disease, but there are significant drawbacks to each. Dialysis can prolong survival via replacement of filtration functions, but other kidney functions are not replaced, thus leading to long-term consequences, such as anemia and malnutrition (Chazan *et al.*, 1991). Currently, renal transplantation is the only definitive treatment that can restore the entire function of the kidney, including filtration, and production of erythropoietin and 1,25 dihydroxyvitamin D3. However, transplantation presents with several limitations, such as a critical donor shortage, complications due to chronic immunosuppressive therapy, and graft failure (Cohen *et al.*, 1994; Ojo *et al.*, 2000, 2001).

Over the last decade, stem cells and their possible role in the construction of bioartificial organs, such as the kidney, have been an area of intense research. In 2007, Perin *et al.* showed that AFSC could be induced to differentiate into renal cells when placed into an *in vitro* embryonic kidney environment (Perin *et al.*, 2007). Human AFSCs

were obtained from human male amniotic fluid, and were labeled with either LacZ or green fluorescent protein (GFP) so that they could be tracked throughout the experiment. These labeled cells were microinjected into murine embryonic kidneys (12.5 to 18 days gestation), and maintained in a special co-culture system *in vitro* for 10 days. Using this technique, it was shown that the labeled hAFSCs remained viable throughout the experimental period, and, importantly, they were able to contribute to the development of various primordial kidney structures, including the renal vesicle, C-, and S-shaped bodies. Studies using reverse transcriptase polymerase chain reaction (RT-PCR) indicated that the implanted hAFSCs began to express early kidney markers such as zona occludens-1 (ZO-1), glial derived neurotrophic factor, and claudin. Together, these data suggested that hAFSCs have the intrinsic ability to differentiate into a number of different cell types that make up the kidney, and could represent a potentially limitless, ethically-neutral source of cells for renal tissue engineering.

IN VIVO BEHAVIOR OF AMNIOTIC FLUID STEM CELLS

Our group has cultured amniotic fluid stem (AFS) cells in neuronal differentiation medium for a time, and then grafted them into the lateral cerebral ventricles of control mice and the ventricles of the *twitcher* mouse model, in which a progressive loss of oligodendrocytes leads to massive demyelination and neuronal loss (De Coppi *et al.*, 2007). AFS cells integrated into the brains of both strains seamlessly, appeared morphologically indistinguishable from surrounding mouse cells, and survived efficiently for at least two months. Interestingly, more of the AFS cells integrated into the injured twitcher brains (70%) than into the normal brains (30%), hinting at the potential for central nervous system therapies. In this study, the phenotypes of the implanted human cells were not assessed. However, the pattern of incorporation and morphologies of cells derived from the AFS cells appeared similar to those obtained previously in the same animal model after implantation of murine neural progenitor and stem-like cells. In that case the donor-derived cells were identified as astrocytes and oligodendrocytes.

From a tissue engineering perspective, osteogenically-differentiated AFS cells were embedded in an alginate/collagen scaffold, and implanted subcutaneously into immunodeficient mice. By 18 weeks after implantation, highly-mineralized tissues and blocks of bone-like material were observed in the recipient mice using micro CT. These blocks displayed a density somewhat greater than that of mouse femoral bone. This indicates that AFS cells could be used to engineer grafts for the repair of bone defects.

FUTURE DIRECTIONS

Much interesting work remains to be done with this cell population. *In vitro*, there remain a few cell types that have not been investigated, but are of great interest scientifically and therapeutically. *In vivo* work to complement the *in vitro* differentiations demonstrating the capacity of these cells to functionally supplement normal tissue will highlight the true clinical potential of these cells. Of great interest may be cell types traditionally senescent in differentiated form, as these cells could be expanded in an undifferentiated form and differentiated into the cell type of interest in large numbers. These progenitor cells, if seeded on a scaffold, could potentially be differentiated into the desired cell types. Such a mixture of correctly differentiated cells could process local cues to structure themselves into highly complex formations in the scaffold much as they do during development. For such reasons, the amniotic progenitor cells may also have practical use in tissue engineering of organs.

The ease of maintenance, proliferation, and differentiation of the amniotic progenitor cells also provides great promise as potential cells that could be used for other purposes such as investigation into development pathways or drug screening. Many experiments to probe the exact potential of these cells and fully characterize their source will be beneficial, as they will help to define realistic goals and applications for use of these cells.

SUMMARY

The pluripotent progenitor cells isolated from amniotic fluid present an exciting possible contribution to the field of stem cell biology and regenerative medicine, and may be an excellent source for research and therapeutic applications. The embryonic and fetal progenitor cells have better potential for expansion than adult stem cells. For this reason, they could represent a better source for therapeutic applications in which large numbers of cells are needed. The ability to isolate the progenitor cells during gestation may also be advantageous for babies born with congenital malformations. Furthermore, the progenitor cells can be cryopreserved for future self-use. When compared with embryonic stem cells, the progenitor cells isolated from amniotic fluid have many similarities: they can differentiate into all three germ layers, they express common markers, and they preserve their telomere length. However, the progenitor cells isolated from amniotic fluid have, in our opinion, considerable advantages. They differentiate easily into specific cell lineages, they do not need feeder layers to grow, and they do not require the sacrifice of human embryos for their isolation, thus avoiding the current controversies associated with the use of human embryonic stem cells. The discovery of these cells has been recent,

and a great deal of work remains to be done on the characterization and use of these cells. Initial results have been promising and are sure to lead to interesting developments.

KEY WORDS

Karyotype The chromosome characteristics of an individual cell or of a cell line, usually presented as a systematized array of metaphase chromosomes from a photomicrograph of a single cell nucleus.

Pluripotent cells Primordial cells that may still differentiate into various specialized types of tissue elements (e.g., mesenchymal cells).

Progenitor cells In development, a parent cell that gives rise to a distinct cell lineage by a series of cell divisions.

Telomere A specialized nucleic acid structure found at the ends of linear eukaryotic chromosomes.

Teratoma Malignant tumor thought to originate from primordial germ cells or misplaced blastomeres that contains tissues derived from all three embryonic layers, such as bone, muscle, cartilage, nerve, tooth buds, and various glands.

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Chapter 17

Cord Blood Hematopoietic Stem and Progenitor Cells

Hal E. Broxmeyer

INTRODUCTION

Cord Blood Transplantation and Banking

October 2008 marked the twentieth anniversary of the first successful umbilical cord blood stem cell transplant, an event celebrated at a number of scientific meetings (see www.cordbloodforum.org; www.eurocord.org; www.esh.org). Since the first cord blood transplant, which cured the disordered and fatal hematological manifestations of Fanconi anemia with human lymphocyte antigen (HLA)-matched sibling donor cells, there have been more than 14 000 cord blood transplants done to treat a wide variety of malignant and non-malignant disorders using partially or fully HLA-matched unrelated and related cord blood. The first and subsequent cord blood transplants using sibling cells were the result of extensive laboratory-based studies, and the first proof-of-principle cord blood bank, established in the author's laboratory, which suggested the feasibility of such transplants with cells previously considered waste material except for some routine clinical testing needs. Presently, this is the first year that numbers of hematopoietic stem cell transplants performed with cord blood have surpassed those done with adult bone marrow. In large part, this increasing use of cord blood as a source of hematopoietic stem cells is due to the quantity and quality of cord blood banks that have developed world-wide, the enhanced transplantation of adults (formerly most cord blood transplants were done in children), as well as enhanced communication between cord blood banks and transplant centers. More than 400 000 cord blood units have been collected and stored in more than 100 cord blood banks.

Advantages and Disadvantages of Cord Blood for Transplantation

Advantages of cord blood as a source of transplantable stem cells include the ready availability of cryopreserved and banked cord blood, as well as the lower incidence of graft-versus-host disease compared to that of bone marrow, allowing the use of cord blood with a greater HLA-disparity than is acceptable for bone marrow transplantation. However, engraftment of neutrophils and, especially, of platelets is delayed after cord blood, compared with bone marrow, transplantation. Limiting numbers of stem–progenitor cells in single collections of cord blood, and the immature nature of these rare repopulating cells, may be responsible for this relatively delayed blood cell engraftment. The use of multiple, unrelated, cord blood units to transplant a single recipient is currently the procedure of choice to alleviate the problem of limiting donor cord blood cell numbers. Attempts to increase cord blood stem cell numbers through *ex vivo* expansion efforts and transplantation of "expanded cells" have not yet resulted in encouraging clinical results, although large efforts are being put forward to learn how to successfully expand human hematopoietic stem cells *ex vivo*.

This chapter focuses on the functional characteristics of cord blood stem and progenitor cells for proliferation, self-renewal, and homing, three important functions for clinical transplantation. Current information on these functional activities of cord blood stem and progenitor cells helps to explain successes with cord blood transplantation. However, efforts to manipulate these cells for enhanced functional activity may prove efficacious in extending the clinical usefulness of cord blood transplantation.

CHARACTERISTICS AND CRYOPRESERVATION OF CORD BLOOD STEM AND PROGENITOR CELLS

Cycling Status and Responses to Growth Factors

Initial laboratory studies suggested that the frequency and proliferative capacity of hematopoietic progenitor cells in cord

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blood was enhanced compared to that found in bone marrow. Cord blood progenitors were in a slow (G0/G1) cell cycle state, but responded rapidly to proliferation-inducing signals from growth factors, including granulocyte–macrophage colony-stimulating factor (GM-CSF), macrophage (M)-CSF, granulocyte (G)-CSF, interleukin (IL)-3, erythropoietin (Epo), thrombopoietin (TPO), potent costimulating cytokines stem cell factor (SCF, also called steel factor), and Flt3-ligand (FL). SCF and FL activate their respective tyrosine kinase receptors c-kit and Flt3 and synergize with many other growth factors, and themselves, to enhance proliferation of cord blood progenitors. SCF, with various CSFs, helped elucidate the enhanced frequency of immature subsets of progenitor cells in cord blood. These proliferative characteristics, as well as *in vivo* studies in mice with the non-obese-diabetic severe-combined immunodeficiency (NOD–SCID) genotype, demonstrated the superiority of cord blood to bone marrow stem cells for engraftment.

Number of Cord Blood Cells Required for Durable Engraftment

Several parameters, including nucleated cellularity and hematopoietic progenitor cell content of cord blood collections, have been used to predict the engrafting capability of these cells. The content of progenitor cells may be a better predictor of speed of engraftment than the nucleated cellularity, although investigators are still seeking means to identify a high-quality cord blood unit for cord blood transplantation. Most clinicians still rely on nucleated cellularity as one of several parameters, including HLA-typing, when determining whether or not to use a specific collection of cord blood for transplantation. A collection in the range of $\geq 2 \times 10^7$ nucleated cord blood cells per kilogram of body weight is the cutoff many transplanters feel most comfortable using, although successful transplants have been reported with collections containing fewer cells.

Cryopreservation of Cord Blood

Almost all cord blood transplants have used cells that were first frozen for cryopreservation and stored in public or private cord blood banks. Thus, the success of cord blood transplantation has relied, and will continue to rely, heavily on the belief that cord blood stem and progenitor cells can be cryopreserved and recovered efficiently in terms of the quantity and quality of the stem and progenitor cells. That this is so was first reported by the author's group for short-term freezes and then for longer-term stored cells. The longest cord blood has been stored frozen and then used for successful clinical transplantation is probably in the 10–12 year or so range. The longest surviving recipient of a transplant with cord blood from a frozen and stored collection has lived more than 20 years post-transplant. That was the recipient of the first cord blood transplant, performed in October 1988.

The most extensive published study demonstrated an average recovery ($\pm$ 1SD) after 15 years storage of defrosted nucleated cells, granulocyte–macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitors, respectively, of 83 $\pm$ 12, 95 $\pm$ 16, 84 $\pm$ 25, and 85 $\pm$ 25. This was based on analysis of the same samples pre- and post-freezing, using the same culture conditions for progenitor cell analysis, and was comparable to the efficiency of recovery of these cells after 10 years of storage. The intact functional capabilities of these defrosted progenitors were highlighted by the extensive proliferative capacities of CFU-GM, BFU-E, and CFU-GEMM, which, respectively, generated colonies of up to 22 500, 182 500, and 292 500 cells after stimulation in a semisolid methylcellulose culture medium with Epo, GM-CSF, IL-3, and SLF. CFU-GEMM colonies could be replated in secondary dishes, with resultant CFU-GEMM colonies as large as those formed in the primary culture dishes. CD34+CD38− cord blood cells isolated from defrosts of cells frozen for 15 years demonstrated more than a 250-fold *ex vivo* expansion of progenitor cells. CD34+ cells isolated from these defrosts were able to engraft NOD–SCID mice with a frequency equal to that of freshly isolated cord blood CD34+ cells. Recent studies in the author's laboratory have demonstrated strikingly similar results with cord blood stored frozen for 22–23 years. Thus, it appears that cord blood can be stored frozen for at least 22–23 years, with a high likelihood that they will be able to engraft human recipients. Although cryopreservation efforts have been relatively successful, studies continue to optimize methods for the cryopreservation of cord blood stem cells.

CORD BLOOD TRANSPLANTATION PROBLEMS AND POSSIBLE COUNTERMEASURES

Background to Problems

Most original cord blood transplants were done in children, due to limiting numbers of cells present in single cord blood collections. More recent emphasis is on the use of double cord blood transplants for treatment. Efforts to enhance the clinical utility of cord blood, both for children and adults, will likely benefit from a greater understanding than is currently available of the self-renewal, proliferation, and homing characteristics of hematopoietic stem cells in general, and of cord blood stem cells in particular. Information in these areas will clearly enhance prospects for successful *ex vivo* expansion of stem cells and for engrafting capabilities of these cells, as well as for nonexpanded stem cells.

Genomics and Proteomics of Cord Blood Stem–Progenitor Cells

A comprehensive genomic and proteomic profile of cord blood, compared to that of adult bone marrow and mobilized peripheral blood, could shed light on the proliferative, self-renewal, and homing potentials of stem cells, and the advantages and disadvantages of each tissue source of these cells. Genomic and proteomic profiling of stem cells has been reported for cells from several sources, including cord blood. However, these reports are limited to CD34+ cell populations. A problem inherent in analysis of CD34+ cells is that they are not a pure population of stem cells; they contain progenitor cells, and the stem cell content is a minor proportion of the total population. Also, this fraction is composed of more than stem and progenitor cells. Even the more highly purified population of human CD34+CD38− cells is not close to being pure in stem cell content. In fact, only about one in 600 CD34+CD38− cord blood cells engraft NOD–SCID mice. Unfortunately, the field of phenotypic characterization of human stem and progenitor cells is not at the level of that obtained for murine stem and progenitor cells. Genomic and proteomic information on human stem cells has to be interpreted cautiously, with the understanding that what is detected may not be specific for, or even present in, the stem cell population. Presently, we must rely on functional analysis of the responses of cord blood stem cells for self-renewal, proliferation, and homing.

Ex Vivo Expansion

Current Knowledge

Although cord blood progenitor cell populations have been extensively expanded *ex vivo* by many different investigators, it is not yet clear that human long-term marrow repopulating stem cells have been expanded much, if at all. It may be relevant in certain circumstances to use *ex vivo*-expanded short-term repopulating stem or hematopoietic progenitors in clinical settings. However, use of *ex vivo*-expanded stem cells will likely require a greater understanding of the self-renewal process of stem cells, especially in the context of the microenvironmental niche in the bone marrow that nurtures these cells. We are just beginning to understand cytokines and intracellular signaling events that influence the self-renewal process of stem cells, but even this understanding is not in context of the stem cell niche.

Cytokines and Intracellular Molecules Implicated in Self-renewal of Stem Cells

Numerous cytokines and chemokines and their receptors have been identified that act alone, and together, to modulate proliferation of myeloid progenitor cells. Information on cell cycle regulation and cell cycle checkpoints is also accumulating for progenitor cells.

A number of cytokines and intracellular signaling molecules have been implicated in proliferation, self-renewal, or both, of hematopoietic stem cells. These include, but are not limited to, the following ligands and their receptors: SCF/c-kit, FL/FLT3, Notch ligands–Notch, and Wnt3a–Frizzled. Intracellular molecules implicated include the following: p21cip1/waf1, p27kip1, p18Inc4c, Hoxb4–Pbx1, Bmi1, Stat3, and Stat5. Nanog, Oct4, Stat3, Hex, and Sirt1, among other signaling molecules, have been implicated in the growth and differentiation of embryonic stem cells. It is possible that intracellular molecules involved in the regulation of embryonic stem cells also play a role in the proliferation, self-renewal, or both, of hematopoietic stem cells. Stat3 has been linked to *in vivo* regulation of hematopoiesis, and serine phosphorylation of Stat3 has been linked to proliferation of progenitor cells in response to combined stimulation by SLF and GM-CSF or IL-3. Overexpression of Hex is associated with enhanced proliferation of myeloid progenitor cells. Stat5 is crucial for FL synergistic stimulation of myeloid progenitor. In fact, the finding that certain intracellular signals, such as Oct4, Sox2, c-Myc, and KLF, allow for reprogramming of mature cells into an embryonic cell-like state may also be of use to understanding functioning of hematopoietic stem cells.

Other cytokines that may influence stem cell proliferation, self-renewal, or both, are TPO, Oncostatin M, and IL-20. TPO, an early-acting cytokine, has been implicated in hemangioblast development and is one of the ingredients, with SLF and FL, used by investigators to *ex vivo* expand hematopoietic stem and progenitor cells. Oncostatin M is a T-helper cell 1 produced cytokine that regulates progenitor cell homeostasis. IL-20 is a candidate stem cell effector molecule that has selectivity for CFU-GEMM among myeloid progenitor cells. IL-20 enhances numbers of CFU-GEMM from human and mouse bone marrow and human cord blood in the presence of SLF and Epo *in vitro.* It has no effect *in vitro* on erythroid, granulocyte–macrophage, or megakaryocyte progenitors. IL-20 transgenic mice have increased numbers and cell cycling of CFU-GEMM, but not of other myeloid progenitors. The administration of IL-20 to normal mice significantly increases only CFU-GEMM numbers and cell cycling. This is the first cytokine reported with such specificity.

Cell Cycle Checkpoints, Asymmetry of Division, and Self-renewal

Self-renewal of hematopoietic stem cells is a poorly understood event. Little is known of the mechanisms mediating stem cell self-renewal. Self-renewal requires cell division without loss of stemness and pluripotentiality in at least one of the daughter cells. This is sometimes referred to as asymmetric cell division. Maintenance, expansion, or loss of stem

cells will depend on the populations of stem cells produced or lost through symmetric or asymmetric divisions. The process by which cell fate determinants are segregated at cell division depends on polarization and segregation across the mitotic spindle; daughters of cell division can inherit similar or very different cellular contents. Proper functioning of the mitotic spindle and its relative positioning is extremely important for regulation of self-renewal, and may be dependent on the microenvironmental niche in which the stem cell resides. Cell cycle checkpoints are necessary to maintain the progression of cell division events in a linear and ordered fashion. Several cell cycle checkpoints have been described. The mitotic spindle assembly checkpoint (MSAC) ensures that the cell cycle does not progress from metaphase to anaphase until all paired sister chromatids are arranged properly across the metaphase plate. This alignment establishes the plane of division. It also establishes the plane of polarity necessary for cell-fate determinant segregation (e.g., self-renewal or differentiation). The MSAC is probably critical for proper regulation of self-renewal and differentiation, and mitotic checkpoint proteins may be involved.

The cyclin-dependent kinase modulator, p21cip1/waf1, has been implicated in the SCF synergistic stimulation of the proliferation of progenitor cells, and in the functioning of stem cells. Synergistic stimulation of cells by combinations of cytokines is likely important to the *in vivo* functioning of both progenitor and stem cells. Of the cytokines that influence stem cell proliferation, self-renewal, or both, all have been shown to work only or more efficiently when used with other cytokines, such as SCF. P21cip1/waf1, because of its role in SCF synergy, could be, and is, important for stem cell function. Because p21cip1/waf1 has been linked to proper functioning of the MSAC, it seems reasonable that the MSAC is involved in the proliferation, self-renewal, or both, of stem cells.

Recently Sirt1, a member of the sirtuin family of deacetylases, has been found to regulate apoptosis and nanog expression of mouse embryonic stem cells by controlling subcellular localization of the p53 tumor suppressor. Sirt1 is involved in the aging process, and may also be relevant to stem cell function and stem cell aging.

In addition to effects on growth, cytokines have been implicated in the survival–antiapoptosis of hematopoietic stem and progenitor cells. One such molecule is a cysteine, another amino acid, cysteine (CXC) motif chemokine, stromal cell-derived factor-1 (Sdf1)/Cxcl12, which signals and induces its activities mainly through the receptor Cxcr4. It is possible that (Sdf1)/Cxcl12 augments replating capacity and *ex vivo* expansion of hematopoietic progenitor cells, and may increase the *ex vivo* expansion of cord blood stem cells when used with one or more of the stem cell active growth factors.

Implications of Self-renewal

Should any of the described growth factors, survival enhancing factors, or intracellular signaling molecules recently implicated in the regulation of hematopoietic, embryonic, or induced pluripotent stem cells prove useful in *ex vivo* expansion of hematopoietic stem cells, such activities may allow enhanced usefulness and broadness of applicability of cord blood transplantation.

HOMING OF STEM AND PROGENITOR CELLS

Importance of Homing

A potential problem associated with *ex vivo*-expanded hematopoietic stem cells may be changes in the characteristics of the homing receptors, adhesion molecules, or both, on these cells that could occur during their time in cell culture. Assessment of the engrafting capability of *ex vivo*-cultured stem cells may underestimate stem cell numbers and activity in a non-obese diabetic–severe combined immunodeficiency (NOD–SCID) or other immune-deficient mouse model for human stem cells. Newer methods that use direct injection of the human cells into the marrow, rather than intravenous injection, may allow the detection of stem cells that cannot accurately home to the marrow. Such intrafemoral transplantation of NOD–SCID mice revealed a short-term repopulating human cord blood cell with a unique phenotype. Although this modification of the NOD–SCID human stem cell repopulating assay may allow a more accurate quantitation of the numbers of stem cells in the test inoculation, the lack of appropriate homing capacity in the NOD–SCID mice might indicate a potential problem with the homing capacity of these cells in human clinical transplantation. Clinical studies evaluating the effectiveness of intra-bone marrow injections of cord blood cells in comparison to intravenous injection have not yet demonstrated the superiority of the intra-bone marrow injection route. Greater insight into homing capacities of hematopoietic stem cells and mechanisms involved could allow enhanced transplantation of donor cells in human recipients.

Sdf1/Cxcl12–Cxcr4 and CD26 in Homing of Stem Cells

It has been recently reported that purified hematopoietic stem cells home with absolute efficiency in lethally-irradiated mice. This, however, is not consistent with the work of several groups, or of actual clinical transplantation results. Perhaps only a subset of stem cells home with absolute efficiency. It is possible to greatly increase the homing and engrafting capacity of long-term marrow competitive engrafting and self-renewing mouse bone marrow stem cells by decreasing the dipeptidylpeptidase IV (DPPIV) activity of CD26, or by eliminating CD26, on hematopoietic stem and progenitor cells, and other cells. DPPIV truncates Sdf1/Cxcl12 into an inactive form that does not have chemotactic

activity, but that can block the chemotactic activity of full-length Sdf1/Cxcl12. The Sdf1/Cxcl12–Cxcr4 axis has been implicated in chemotaxis, homing, and mobilization. Either inhibiting CD26 peptidase activity with inhibitors such as Diprotin A or Val-Pyr, or functionally deleting CD26, results in greatly reduced G-CSF-induced mobilization of progenitor cells. It is believed that the mechanism of this latter effect is caused by the inactivation or elimination of CD26, which blocks the truncating effect of CD26 on Sdf1/Cxcl12, thus allowing stromal cell-produced Sdf1/Cxcl12 to maintain a greater "holding" action on Cxcr4-expressing stem and progenitor cells.

Based on this belief, it was hypothesized that inactivation or elimination of CD26 on stem cells or other cells might enhance the homing capacity of exogenously infused stem cells. Experimental evidence supported this hypothesis as Diprotin A- or Val-Pyr-treated congenic wild-type mouse marrow cells or marrow cells from CD26−/− mice demonstrated short-term homing and long-term marrow competitive repopulating capacities of these stem cells greater than the capacities of wild-type marrow cells treated with control medium or the capacities of untreated CD26+/+ marrow cells. It is possible that the enhanced marrow repopulating capability of stem cells in which CD26 is inactivated or eliminated may result partly from the enhanced stem cell survival activity of nontruncated–inactivated Sdf1/Cxcl12. Treatments such as inactivation or loss of CD26 activity that have the capacity to enhance the homing–engrafting capability of hematopoietic stem cells may allow greater engrafting capability with limiting numbers of stem cells, such as found in cord blood, effects reported for human CD34+ cord blood cells engrafting sub-lethally irradiated NOD–SCID mice, and may also enhance the homing–engrafting capability of *ex vivo*-expanded stem cells. Inhibition of CD26 on mouse bone marrow and human cord blood cells also enhances the specific activity of growth factors/CSFs that contain a putative CD26 truncation site. Thus, inhibition of CD26 may act in multiple ways to enhance engrafting capability of hematopoietic stem cells.

SUMMARY

The frequency and quality of hematopoietic stem cells in cord blood at the birth of a baby have endowed cord blood with the capacity to cure a large variety of malignant and genetic disorders. Cord blood transplantation works in both children and adults. Being able to *ex vivo* expand, and enhancing the homing efficiency, of stem cells from cord blood would increase the usefulness and applicability of cord blood for transplantation. Although attempts at *ex vivo* expansion of stem cells for clinical cord blood transplantation have been disappointing thus far, new information regarding factors and intracellular signaling molecules involved in the regulation of hematopoietic stem cell activity, the role of the microenvironmental niche in this regulation and in the growth of embryonic stem cells and the newly identified field of inducible pluripotent stem cells (which may be translatable to hematopoietic stem cells), as well as advancements in understanding and manipulating the homing capacities of stem cells, offer hope that we may soon be ready to enhance stem cell transplantation in general and cord blood stem cell transplantation in particular.

KEY WORDS

CD26/Dipeptidylpeptidase IV (DPPIV) An enzyme that truncates and inactivates certain chemokines, including Sdf1/Cxcl12, and selective growth factors (e.g., cytokines). Truncated chemokines/cytokines block activity of full length chemokines/cytokines. Thus, inhibition of CD26/DPPIV enhances the specific activity of chemokines/cytokines with this truncatable site in the context of active CD26/DPPIV.

Chemokine A family of cytokines originally defined by their chemo-attractant properties, but having additional functional activity on cells. An example is SDF-1/CXCL12, involved in movement and survival of cells.

Cord blood transplantation Infusion of umbilical cord blood containing hematopoietic stem and progenitor cells into recipients to replace the blood cell system.

Cryopreservation The means to preserve cells long-term by placing them at very low temperature for prolonged periods of time, in order to eventually defrost and recover the cells in a full physiologically and metabolically active state.

Cytokine Biologically active molecule that occurs naturally and influences the survival, proliferation, migration, or other aspects of immature or mature cells.

Hematopoietic progenitor cell The progeny of a stem cell that has little or no self-renewal capacity, but is committed to produce mature blood cell types of one or more lineages (e.g., granulocytes, macrophages, erythrocytes, megakaryocytes, lymphocytes, etc.).

Hematopoietic stem cell A cell that can reproduce itself (self-renew) and give rise to all the blood-forming tissue.

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Chapter 18

Neurogenesis in the Vertebrate Embryo

Chris Kintner and Naoko Koyano-Nakagawa

INTRODUCTION

The recent establishment of human embryonic stem cells (hESCs) has fueled the idea that restricted progenitors or differentiated cell types can be generated *in vitro* from these pluripotent cells, and then used in a therapeutic setting to repair human tissue damaged by trauma or disease. hESCs successfully converted into the appropriate neural cell types might be used, for instance, to repair neural tissue that is damaged in such human conditions as Multiple Sclerosis, Parkinson's, Alzheimer's, Amyotrophic Lateral Sclerosis, or spinal cord injury. Given this goal, one approach is to apply differentiation protocols to hESCs that mimic the extrinsic and intrinsic cues that underlie the formation of neural cell types in the embryo. Indeed, one of the more successful protocols for differentiating mouse ES cells into transplantable motor neurons is based on a systemic understanding of the developmental mechanisms that underlie the formation of the embryonic spinal cord. Similar logic has also guided subsequent attempts to differentiate ES cells into other specific classes of neurons, including retinal photoreceptors, cerebellar granular neurons, cerebral-type neurons that use glutamate, GABA or dopamine as their principle neurotransmitter. With this approach in mind, the goal of this chapter is to provide a brief overview of how neural progenitors first arise during embryonic development, and how the fate of these progenitors are determined so that they give rise to the diverse types and number of neurons that comprise different regions of the vertebrate central nervous system (CNS). A comprehensive review of the developmental mechanisms underlying these processes is beyond the scope of this chapter. Accordingly, we will illustrate general principles by focusing on the spinal cord and the formation of neural precursors that give rise to a specific neuronal subtype, the motor neuron (MN).

EMBRYONIC INDUCTION AND THE ESTABLISHMENT OF NEURAL TISSUE

Embryonic cells acquire a neural potential soon after gastrulation, when ectoderm (also known as the epiblast) forms the neural plate on the dorsal side of the embryo (Figure 18-1). The neuroepithelial cells (NECs) of the neural plate subsequently form a tube arranged around a central lumen that extends along the anterior–posterior axis (A–P) of the embryo (Figure 18-2A, B), thus creating progenitors that divide and subsequently differentiate into three major cell types that comprise the CNS, namely neurons, myelinating oligodendrocytes and supporting astroglia. For simplicity, we will refer to these restricted embryonic precursors as NECs, although they are often referred to as neural progenitors or neural stem cells. At the same time the neural plate appears, regions of ectoderm located more ventrally in the embryo form other important progenitor cell populations, including the neural crest, migrating progenitors that give rise to both neural and nonneural cell types, and placodal structures that contain neural precursors for sensory ganglia, as well as the neurons in the ear and nose. Finally, the most ventrally located ectoderm forms precursors that give rise to non-neural tissue, most notably skin. Classical transplantation experiments in the early 1900s using amphibian embryos revealed the importance of inductive interactions for dividing the ectoderm into these different regions. For example, while ectoderm isolated away from the embryo only forms skin, the same tissue can be induced to form a complete nervous system through interactions with a region of the embryo called the organizer. Subsequently this process, called neural induction, was described in chick and mouse embryos, and is now known as the key event in establishing neural progenitors in all vertebrates, including, by extension in human embryos.

MOLECULAR BASES OF NEURAL INDUCTION

Using the assays established by embryologists, the molecular bases of embryonic induction became an active area of research in the 1990s. The results that emerged from these studies emphasized the importance of signaling by different families of growth factors, including members

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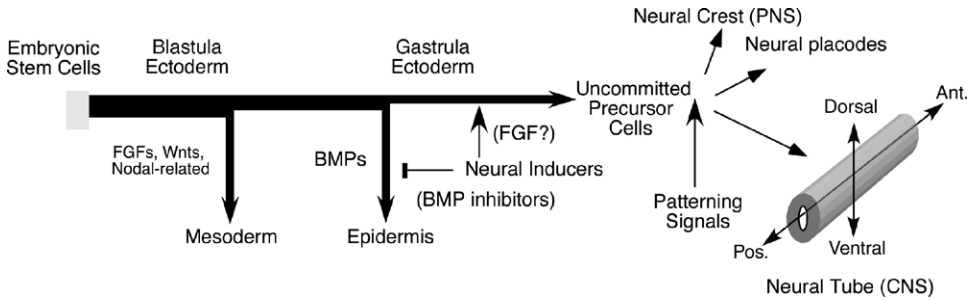


FIGURE 18-1 Default model of neural induction. Following fertilization, a region of the early embryo gives rise to the ectoderm or epiblast, which responds to inductive signals as development progresses from left to right. At blastula stages, these signals include ones that induce mesodermal derivatives in posterior regions of the embryo, via growth factors such as FGF, Wnts, and nodal-related families. At gastrulae stages, ectoderm on the ventral side is induced to become epidermis by BMP signaling. However, a region of ectoderm avoids BMP signaling via inhibitors produced by the organizer, giving rise to neural tissue. This neural tissue responds to a variety of patterning signals that divide it into different neural fates.

of the TGF-β, the fibroblast growth factor (FGFs), and the Wnt superfamilies. Growth factor signaling first comes into play before gastrulation, when it plays a critical role in the induction of mesodermal derivatives. Thus, exposing embryonic cells of the blastula to the nodal-related members of the TGF-β superfamily or to FGFs prior to gastrulation induces these cells to give rise to mesodermal derivatives such as muscle, blood, or notochordal cells (Figure 18-1). Embryonic cells then lose the competence to respond to mesoderm induction at the start of gastrulation when other members of TGF-β superfamily, the BMPs, become pivotal in specifying cell fate. BMPs, such as BMP4 and BMP2, are produced by the ectodermal cells during gastrulation and specify a ventral or epidermal fate. By contrast, BMP signaling in dorsal ectoderm is inhibited extracellularly by molecules such as noggin, chordin, and follistatin which are produced by the organizer, thereby allowing dorsal ectodermal cells to avoid the epidermal fate and give rise instead to neural tissue. Thus, embryonic cells appear to acquire neural potential if they avoid a series of signaling events that promote their differentiation along non-neural lineages, a process called the default model for neural induction (Figure 18-1).

The molecular analysis of embryonic induction established several important concepts in terms of how embryonic cells take on particular fates in response to growth factor signaling. The first is that the response of embryonic cells to a particular growth factor is highly context-dependent. Embryonic cells, for example, will form mesoderm in response to nodal-related signaling for only a brief period before losing this response, or responding in a different way. Second, the nature of response of embryonic cells to growth factor signaling is often concentration-dependent, allowing cells to take on different fates when exposed to different levels of the same factor. Finally, the analysis of neural induction revealed the importance of mechanisms that inhibit growth factor signaling in cell fate decisions. Among the most potent neural inducers, for instance, are the extracellular inhibitors of BMP signaling. Thus, guiding the fate of embryonic cells into a neural lineage may be largely dependent on the timing and dose of different growth factors, as well as the presence of inhibitors that block signaling by other growth factors.

When ectodermal cells acquire a neural potential, they activate the expression of critical transcription factors that generally mark neural progenitors, such as Sox2. Sox2 not only marks, but also maintains NECs as neural progenitors, as discussed further below. Based on the default model (Figure 18-1), inhibiting BMP signaling may be necessary for Sox2 to be expressed, but still leaves open the possibility that other positive factors are required to activate and maintain Sox2 expression, thereby inducing a neural plate identity. Currently, our understanding of these positive factors is relatively limited, with one exception being FGF signaling acting through the MAPK pathway. In chick experiments, FGF is much more effective than the extracellular BMP inhibitors at inducing neural tissue in epiblast cells adjacent to the neural plate. In frog embryos, extracellular BMP inhibitors are relatively ineffective at neuralizing ectoderm on the extreme ventral side of the embryo, unless FGF signaling is also active. Finally, FGF signaling is required to maintain neural precursor cells in culture, and for regulating their differentiation *in vivo*. While these observations seem to contradict the default model, it should also be kept in mind that the signaling components downstream of the FGF receptor can intersect with BMP signaling, by phosphorylating and inactivating a critical downstream target of BMP signaling, the Smad proteins, or by activating the expression of SIP1, a negative regulator of BMP target genes. Thus, FGF and perhaps Wnts may contribute to neural induction by dampening the levels of BMP signaling, while also promoting BMP independent events that neuralize embryonic cells. Even with this better understanding of the signals that underlie neural induction, a major challenge is to determine how embryonic cells respond by activating the genes they need as neural progenitors.

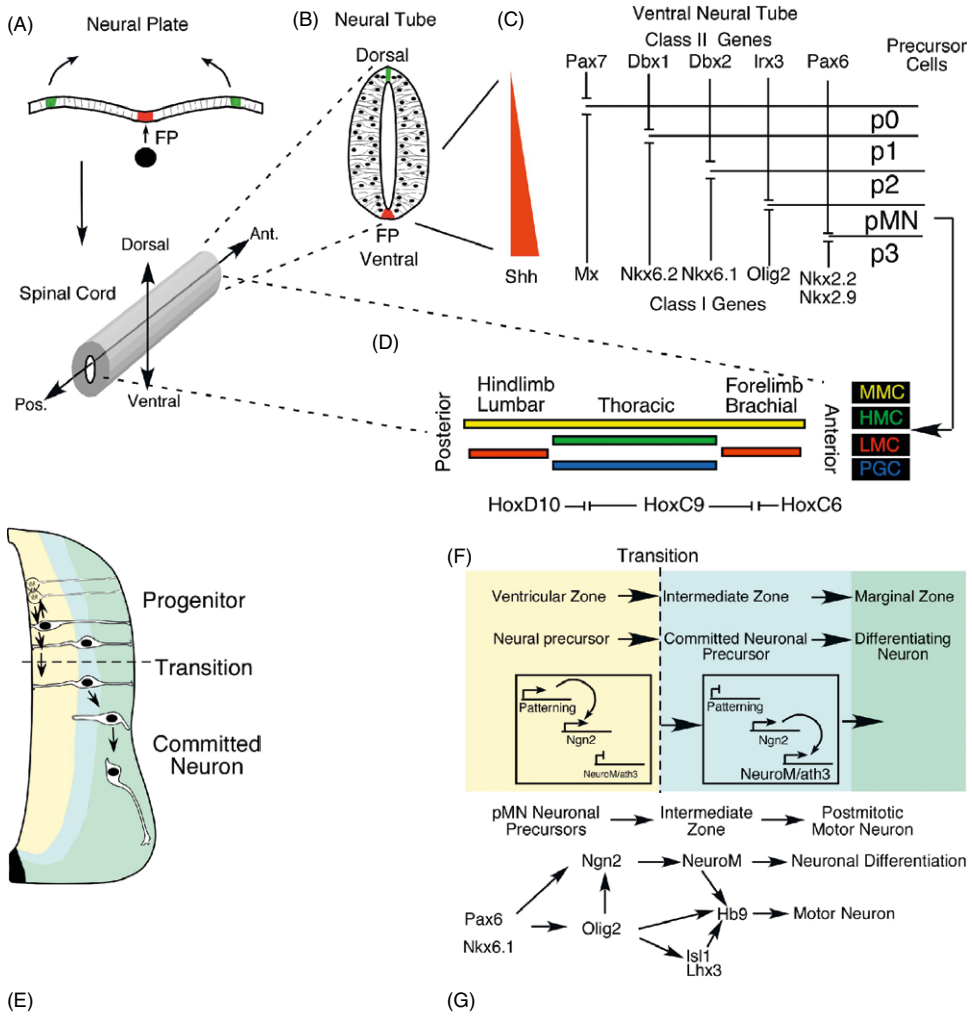


FIGURE 18-2 Patterning and neurogenesis in the ventral spinal cord. (A) The neural plate forms during gastrulation as a thickening of the ectoderm into a neuroepithelium, at which time patterning along the dorsal ventral axis begins with the establishment of the floor plate (red shading, FP) and the roof plate (green shading). (B) As the neural plate forms a neural tube, the NECs are patterned by signals emanating, in part, from the floor and roof plates. (C) In ventral spinal cord, this patterning consists of a gradient of Shh secreted by the floor plate, which activates the expression of the class I homeodomain proteins (one exception is Olig2 which is a bHLH repressor) in a concentration-dependent fashion. The class I proteins interact with the class II proteins to divide the NECs into different regions of neural precursors. Mx is a hypothetical protein that has been proposed to contribute to ventral patterning by analogy with other class I proteins. (D) Patterning of the spinal cord along the A–P axis sets in place nested expression of other transcription factors that intersect with those along the D–V axis to establish neuronal fate. The best studied example of this mechanism is how A–P patterning of the pMN domain allows this progenitor to give rise to motor neurons with different columnar identities. Indeed, the MNs in the spinal cord fall into different columns that localize differentially along the A–P axis and innervate different peripheral targets. The medial motor column (MMC) innervates axial muscle and is present along the entire A–P axis. Motor neurons in both the preganglionic motor column (PGC) and hypaxial motor column (HMC) are only present at thoracic levels, and innervate the para- and sympathetic chain or body wall muscle, respectively. Finally, motor neurons in the lateral motor column (LMC) are only present at limb levels and only innervate the limbs. The specification of the motor neurons into these different columns is controlled, in large part, by genes in the HOX complexes, as indicated, and whose expression is established by A–P patterning. (E) Neurogenesis within the neural tube is organized along a third developmental axis that corresponds to the apical–basal orientation of the neuroepithelium. Progenitor cells consist of NECs localized along the apical surface of the ventricular zone (yellow shading), within which their nucleus transverses during the cell cycle. During cell division, the two daughter cells separate at the apical surface, with recent studies suggesting that they maintain contact with the basal surface. A precursor undergoes neuronal differentiation when its nucleus migrates laterally (blue zone), exiting the cell cycle. Terminal neuronal differentiation is likely to be completed when a precursor delaminates from the neuroepithelium by detaching from the ventricular surface, and migrating laterally into the marginal zone (green shading). (F) Expression of proneural genes during the different phases of neurogenesis as shown by color coding used in panel E. Proneural genes, such as the neurogenins, are expressed in dividing precursors within the ventricular zone (yellow shading). Neuronal commitment occurs in the intermediate zone (blue shading) when the levels of neurogenin are sufficiently high to activate the expression of downstream proneural genes. Thus activation of the proneural cascade results in a commitment and the transition to a postmitotic neuron (blue shading) that undergoes terminal neuronal differentiation (green shading). (G) Integration of neuronal differentiation with neuronal subtype specification. In ventral spinal cord, patterning leads to expression of both the proneural protein Ngn2 and HD transcription factors, which cooperate to activate the expression of the motor neuron determinate called HB9. This cooperation integrates a program of neuronal differentiation promoted on the one hand by Ngn2, and perhaps Ath3/NeuroM, along with a program of motor neuron differentiation.

NEURAL PATTERNING

The role of multiple factors, such as the BMP inhibitors and FGF, in neural induction could be an indication that neural precursors for different regions of the nervous system arise by different mechanisms. Indeed, even though they might appear morphologically homogeneous as they form the neural plate, neural progenitors already differ markedly, based on gene expression according to their position along both the D–V and A–P neuraxis. In this view, neural precursors are never generic in nature, but rather form as distinct populations based on their position. Indeed, as NECs form the neural plate, they are exposed to a variety of signals that specify their position within the nervous system along two major cardinal axes. Understanding how NECs acquire positional information is critical in any strategy that is designed to generate progenitors capable of giving rise to neurons that are appropriate for a given region of the nervous system.

Specification of NECs along the A–P neuraxis remains poorly understood, but is thought to involve posteriorizing signals in the form of the Wnts, retinoic acid (RA) and FGFs. These factors, acting in combination with the BMP inhibitors described above, are thought to convert NECs from an anterior ground state to a more posterior position along the neuraxis, although the details of this process remain unclear. For the spinal cord, however, exposure of neural progenitors to gradients of FGF and RA signaling is likely important for establishing A–P axial position, by activating the expression of genes in the HOX complexes, as discussed further below in the context of motor neuron specification.

Specification of position along the D–V axis of the spinal cord is much better understood and, indeed, has become the paradigm for how NEC position is determined in the CNS. D–V patterning of the spinal cord depends largely on signals produced by two specialized midline structures, one at the ventral pole of neural tube (the floorplate and underlying notochord) and the other at the dorsal pole (the roof plate) (Figure 18-2B). Signals produced by these so-called organizing centers subdivide the NECs of the neural tube into domains with different developmental fates. Specifically, the ventral organizing center secretes Sonic hedgehog (Shh, Figure 18-2B), a morphogen that induces or suppresses, at different concentration thresholds, the expression of genes, usually ones encoding homeodomain (HD) transcription factors, in NECs lying at different positions in the ventral spinal cord (Figure 18-2C). In this manner, the gradient of Shh activity subdivides the ventral NECs into at least five distinct areas by activating the expression of different HD transcription factors, as well as one bHLH transcription factor called Olig2, which then sharpen into non-overlapping zones by cross-repression (Figure 18-2C). NECs in one of these zones (pMN, Figure 18-2C) produce motor neurons, while those in the other zones produce various classes of interneurons. Significantly, the response of NECs to Shh occurs over time and is dynamic in nature, due to feedback changes in both negative and positive components in the Shh signal transduction pathway. These dynamic changes are thought to be important in creating a robust system, so that a reproducible outcome in terms of HD and *Olig2* gene expression is achieved, even if the initial gradient of Shh production is less precise.

The patterning events along the D–V neuraxis intersect with those along the orthogonal A–P neuraxis, thereby generating a Cartesian coordinate system that specifies the fate of NECs. An example of how this code operates comes from the analysis of the progenitors that give rise to MNs. As described above, patterning along the D–V axis specifies an MN progenitor domain that forms along the entire A–P length of spinal cord. However, the MNs that arise from this domain can differ along the A–P neuraxis, based on their peripheral target. For example, the progenitors located at limb level are able to give rise to MNs that form the lateral motor column and innervate the limbs, while those located at intervening thoracic levels are able to give rise to MNs that form the hypaxial motor column and innervate body wall muscles. Recent evidence suggests that these different column identities are specified by another set of homeodomain transcription factors, namely those encoded by the HOX clusters. In vertebrates, there are four related clusters (HOXA-D) derived from a common ancestor, each containing around ten genes, with a total of 39 genes in all. Expression of each HOX gene within a cluster is restricted along the A–P embryonic axis, and is determined by its chromosomal location: those at the 3′-end of the cluster have a more anterior boundary of expression than those at the 5′-end of the cluster. This expression pattern, called spatial co-linearity, is set up in the developing spinal cord when it first forms in the embryo during neural tube formation, and is thought to be cued by several signaling molecules, including FGF and RA. As a consequence, the columnar position of developing MNs is marked by differential HOX gene expression along the A-P axis. For example, Hoxc6 is expressed in developing MNs at forelimb levels, Hoxc9 at thoracic levels and Hoxd10 at hindlimb levels (Figure 18-2D). Furthermore, functional studies indicate that this differential Hox gene expression is essential for generating MNs columnar subtype. Thus, D–V patterning of the NECs in the spinal cord establishes motor neuron progenitors via one set of transcription factors, while patterning along the A–P axis enables these progenitors to give rise to MNs with different columnar fates via another set of transcription factors (e.g., Hox genes).

In sum, the neural progenitors in the spinal cord that form during neural induction acquire their fate based on the code of transcription factors that are expressed in NECs as a result of patterning events occurring more or less simultaneously along the D–V and A–P neuraxis. Acquisition of

this transcription factor code by progenitors is critical for the production of a given spinal cord neuron, for example an MN with a given columnar identity.

The Proneural Gene Cascade, a Molecular Switch Underlying Neuronal Differentiation

How, then, does patterning of the NECs discussed above dictate precise patterns of neuronal differentiation? The key finding addressing this question has come from the discovery of a class of bHLH proteins encoded by the so-called proneural genes. As transcriptional activators, the proneural proteins are thought to activate gene expression necessary for both the differentiation of precursors into neurons, as well as for neuronal cell type specification, thus acting as a molecular switch of differentiation (Table 18-1).

The vertebrate proneural bHLH genes fall into two families, based on homology to bHLH genes that were originally identified in *Drosophila* as mutations that block neural differentiation (Table 18-1). One smaller family consists of those related to the *Drosophila* Achaete-Scute genes, such as Ascl1 (formerly called Mash1). The second, larger, family encodes proteins related to *Drosophila* atonal, and can be subdivided structurally into three subfamilies, the neurogenin (Ngn)-like, the NeuroD-like and the atonal-like. Expression of these different subfamilies occurs in precise spatial and temporal patterns, both within the dividing NECs as well as in cells that have initiated neuronal differentiation. When eliminated by targeted mutation, loss of specific proneural bHLH genes results in deletion of specific populations of neurons. However, the loss of neurons is likely to be much more severe when multiple members are simultaneously eliminated, indicating that the proneural genes have overlapping function, as also found in *Drosophila*. Because of this genetic redundancy, it is difficult to test whether all neuronal differentiation is driven by proneural gene action experimentally. However, in gain-of-function experiments, proneural proteins are potent inducers of neuronal differentiation when ectopically expressed, not only in neural precursors, but also in some nonneural tissues.

The proneural proteins function to initiate many of the changes that occur when NECs undergo terminal neuronal differentiation. One such function is to promote cell cycle exit, an irreversible set of events incurred by all NECs as they form neurons. NECs initiate cell cycle exit when their nuclei move to the lateral edge of ventricular zone where they enter the G0 phase and eventually delaminate out of the neuroepithelium (Figure 18-2E). Ectopic expression of the proneural proteins in NECs, or in tissue culture models of NECS, causes rapid cell cycle arrest, although some subtypes of proneural proteins promote this transition better than others. The mechanism by which the proneural proteins initiate irreversible cell cycle exit appears to be quite complex, and is currently an area of active research. This mechanism may involve direct protein–protein interactions with cell cycle machinery, or alternatively transcriptional changes in expression of genes that encode cell cycle regulators, such as cyclin-dependent kinase inhibitors p21, p16, and p27.

Proneural proteins also function to activate the expression of genes associated with all subtypes of neurons, such as those that encode neuronal isoforms of the cytoskeletal proteins, channels involved in membrane excitability, and proteins involved in axon guidance. Significantly, genes encoding the proneural proteins are expressed in neural precursors and in committed neurons, but often are transient in expression and thus lost as neurons mature. Thus, proneural proteins may initiate expression of pan-neuronal genes directly, and then maintain expression indirectly by activating a downstream transcriptional network, as discussed further below.

Another function associated with the proneural proteins during neuronal differentiation is the inhibition of gene expression required for astroglia or oligodendrocyte differentiation. Neural precursors first give rise to neurons and then switch over to produce both types of glia at later stages, suggesting that glial differentiation genes need to be repressed in neural precursors during neurogenesis. Studies using cultured neural stem cells indicate that the proneural proteins inhibit astroglia differentiation in neural precursors, not by binding DNA but rather by competing for critical co-activators required to induce the expression of glial genes such as GFAP. In addition, proneural proteins can also interfere with growth factor induction of glial differentiation by binding to and inhibiting components of the CNTF signaling pathway. Ectopic expression of proneural proteins also suppresses the formation of oligodendrocyte precursors that normally arise within discrete regions of the neural tube after neurogenesis is largely complete. Thus, one function of the proneural proteins is to prevent cells from expressing genes necessary for glial differentiation, while activating those required for neuronal differentiation.

A fourth change associated with proneural activity involves the changes in cell morphology that occur when NECs lose their epithelial characteristics and migrate away from the ventricular zone, in some case for long distances, before elaborating characteristic dendritic and axonal arbors (Figure 18-2E). Proneural activity induces these changes partially by transcriptional regulation of genes involved in neuronal migration, and partially by mechanisms that appear to be non-transcriptional in nature.

Finally, the proneural proteins are not only involved in promoting changes associated with generic neuronal differentiation, they are also involved in activating gene expression required for neuronal subtype specification. Since proneural proteins fall into a number of subfamilies with distinct sequence differences, one possibility is that a given subfamily is specialized to promote the differentiation of

TABLE 18-1 Known Functions of bHLH Proteins in Neuronal Differentiation

Group	Sub-group	bHLH Name	Comments
Acheate-scute	Acheate-scute-like	Mash1/Ascl1	Required for differentiation of autonomic neurons and sub-population of GABAergic interneurons Autonomic and olfactory neuron differentiation Coordination of differentiation in ventral forebrain Determination gene in olfactory sensory neurons Promotes neuronal fate and inhibits astrocytic fate in cortical progenitors Negative indirect autoregulation of the promoter Retinal development
		Xash1	Xenopus homolog of Mash1, expressed in anterior regions of the CNS
		Cash1	Chick homolog of Mash1 and Xash1, has similar expression pattern
		Xash3	Only in Xenopus, early neural plate expression
		Cash4	Only in chick, early proneural gene in posterior CNS
	Nscl-like	Nscl1/NHLH1, Nscl2/NHLH2	Expressed during "late" phases of neuronal committment
Atonal	Neurogenin-like	Xngn1	Promotes neurogenesis in both neuroectoderm and ectoderm Overexpression in developing embyos induces various downstream targets such as Ath3, Xcoe2, Delta, MyT1, NeuroD, Tubulin, Neurofilament, Hes6, XMTGR1/XETOR, and NKL
		Ngn1/ NeuroD3/ Math4C	Sensory lineage Required for proximal cranial sensory ganglia Neurogenesis in developing dorsal root ganglia Specification of dorsal interneurons by cross-inhibition with Math1 Differentiation in olfactory sensory neurons Inhibits gliogenesis
		Ngn2/Math4A	Required for epibranchial placode-derived cranial sensory ganglia Promotes neuronal fate and inhibits astrocytic fate in cortical progenitors Induced by and cross-regulates with Pax6 Promotes cell cycle exit, expression of neuronal markers, induces migration of postmitotic neurons Specifies neuronal cell type in combination with transcription factors involved in patterning
		Ngn3	Promotes gliogenesis in the spinal cord
	NeuroD-like	NeuroD/beta2	Converts Xenopus ectoderm into neurons Cell fate, determination, differentiation, and survival in neural retina Required for differentiation of the granule cells in the cerebellum and hippocampus Survival of inner ear sensory neurons Neurite outgrowth
		NeuroD2/ NDRF	Required for development and survival of CNS neurons

		Math2/Nex1/ NeuroD6	Expressed in postmitotic cells of the brain Induce differentiation of PC12 cells and expression of GAP-43 gene
		Xath2	Expressed in postmitotic cells of stage 32+ Xenopus dorsal telencephalon
		Math3/ NeuroD4/ Atoh3/NeuroM (chicken)	Expressed in postmitotic, premigratory stage in neurogenesis Amacrine cell specification in the retina Cooperates with Lim-HD proteins to specify motorneurons
		Xath3	Converts ectoderm into a neural fate Promotes sensory neuron marker expression
	Atonal-like	Math1	Required for cerebellar granule neuron development Required for generation of inner ear hair cells Required for proprioceptor pathway development Specification of dorsal interneuron sub-population
		Xath1	Expressed in hindbrain Induces neuronal differentiation in ectoderm
		Math5/Atoh7	Promotes retinal ganglion cell fate through brn-3b Retinogenesis, regulated by Pax6
		Xath5	Retinal ganglion cell fate Regulates neurogenesis in olfactory placode
		Math6/Atoh8	Promotes neuronal fate at the expense of glial fate
Olig	Olig-like	Olig1, Olig2	Specification of Motorneuron and Oligodendrocytes Motorneuron specification in combination with ngn2
		Olig3	Transiently expressed in different types of progenitors of embryonic CNS
E protein	E2A	E12/E47	Dimerization partner of various bHLH proteins

a particular type of neuron. Indeed, in *Drosophila* there is strong evidence that the achaete-scute class of proneural proteins induces one type of external sense organ, while the atonal-class induces another. Similar differences have also been described for vertebrate proneural proteins, suggesting that they are designed, in part, to activate different downstream targets associated with neuronal cell type specification. The best understood example of this occurs during the specification of motor neurons via the expression of an HD transcription factor called HB9 (Figure 18-2G). Expression of HB9 is only activated at the point where neural precursors in the ventral neural tube exit the cell cycle and give rise to motor neurons (the Intermediate zone in Figure 18-2E). Analysis of the enhancer required for this activation reveals an element with closely-aligned binding sites for proneural proteins, as well as for two HD proteins, Islet1 and Lhx2, which are known to be required for motor neuron differentiation. Binding of these factors cooperatively activates the expression of HB9, thus driving motor neuron differentiation. Significantly, while some proneural proteins can cooperate to activate HB9 expression, others cannot. In addition, when *Ngn2* activates HB9, it needs to be phosphorylated at key residues to carry out this task, suggesting that cell type specification by the proneural proteins may be regulated by kinase activity.

Similar links have been made between the patterning of NECs and the expression of proneural proteins in the dorsal spinal cord. In this case, neighboring domains of NECs produce different classes of interneurons by expressing distinct members of the proneural bHLH family. Experimentally manipulating the expression of different proneural proteins leads to predicted changes in interneuron fates. Thus, these observations suggest strongly that proneural proteins function to execute generic neuronal differentiation, as well as to activate the downstream target genes needed for neuronal cell type specification.

Potential Links Between Neural Patterning and the Control of Neurogenesis

Because the proneural proteins behave as a molecular "switch" that promotes neuronal differentiation, how their activity is regulated has important consequences for determining the fate of NECs. If, on the one hand, proneural activity is high enough, NECs exit the cell cycle and undergo terminal neuronal differentiation (Figure 18-2F). On the other hand, if activity of proneural proteins is kept low, NECs are maintained as progenitors and, over the protracted period of neurogenesis and gliogenesis, can give rise to later-born neurons or glia at later stages. Not surprisingly, therefore, many of the factors that control the fate of NECs seem to converge on the expression and/or activity of the bHLH proteins, including the patterning genes discussed above.

PRONEURAL GENE EXPRESSION

A conceptually simple means of regulating proneural gene activity is by controlling their transcription. The analysis of the enhancer that drives expression of the proneural gene, *Ngn2* in the spinal cord has revealed a complex number of discrete elements that are responsible for different spatial and temporal expression patterns in NECs. These elements are likely to be driven by transcription factors whose expression is spatially restricted in NECs during neural patterning. For example, in the ventral spinal cord, the first neurons to be generated are motor neurons, and their generation in chick spinal cord is correlated with the early expression of *Ngn2* within a narrow ventral domain of NECs. Expression of *Ngn2* gene at this stage is likely to be promoted in part by the patterning genes that are regulated by Shh, notably Pax6, acting directly as an activator, and Olig2, acting indirectly as repressor (Figure 18-2). For example, when ectopically expressed in the embryonic spinal cord, Olig2 induces ectopic motor neuron differentiation, and does so in part by inducing ectopic and precocious expression of *Ngn2*. Significantly, motor neurons arise in response to ectopic Olig2 with much the same kinetics as they normally do in the ventral neural tube. In addition, Olig2 not only has a positive effect on *Ngn2* expression, it also has an inhibitory effect on its activity by competing for the E-box sites that *Ngn2* binds when activating its downstream targets. This push–pull mechanism may allow Olig2 to establish neuronal progenitors that can expand through division before initiating neuronal differentiation.

The Proneural Cascade

Proneural activity can also be regulated in NECs, by inhibiting their ability to activate their downstream targets. Perhaps the most critical proneural target in this respect is the so-called proneural cascade, where proneural proteins expressed in NECs progenitors are under tight control, but promote differentiation by reaching a threshold that activates a downstream bHLH gene, such as Ath3/NeuroM, which can then mediate the variety of changes associated with neuronal differentiation (Figure 18-2F). As a result, direct or indirect changes in the strength of this cascade constitute one strategy that can be exploited during the process of patterning to control neuronal differentiation. The following discussion reviews some of the prominent regulators of the bHLH cascade that have been described, most of which are inhibitory in nature.

NEGATIVE REGULATORS OF PRONEURAL ACTIVITY

Because the activation of the proneural cascade is a critical step in neuronal differentiation, a variety of inhibitors have

been found that suppress this cascade, thus shaping the onset and extent of neurogenesis. Perhaps the most important inhibitors in this respect are the large family of bHLH transcriptional repressors called Esr, Hes, Her, Hrt, and Hey, depending on their species of origin and subfamily classification of their structure. Many of these genes were isolated and named based on homology to genes in *Drosophila*, called Hairy or Enhancer of Split, which play important roles in inhibiting proneural genes during fly neural development. Functional analysis of these bHLH repressors shows that they potentially antagonize the activity of the proneural proteins by several mechanisms: by direct protein–protein interactions; by competing with the proneural proteins for their binding sites (the E-box) in DNA; or by binding distinct DNA elements (the N-box or high affinity repressor sites) in enhancers targeted by the proneural proteins. Another important class of inhibitors is the Id family: related HLH proteins that contain a dimerization domain but are unable to bind DNA. Since the proneural proteins bind DNA as heterodimers with the ubiquitously expressed E-proteins, they are inactivated when they instead form non-functional dimers with the Id proteins.

Both the bHLH repressors and Id proteins are expressed in NECs, and have been shown to play important roles in determining the pattern and extent of neurogenesis. When these genes are removed by targeted mutations in the mouse, the general phenotype observed is precocious and increased numbers of precursors undergoing neuronal differentiation. Conversely, ectopic expression of these factors in gain-of-function experiments in a variety of experimental systems strongly inhibits neurogenesis.

It is important to emphasize that the Id proteins and bHLH repressors are employed by NECs in several distinct ways to regulate neurogenesis. NECs in some regions of the CNS, such as those at the midbrain/hindbrain boundary, never undergo neurogenesis, in which case they constitutively express bHLH repressors at high levels. Significantly, to achieve such high levels of expression, patterning genes must disable the negative feedback loop that normally causes bHLH repressors to repress their own expression. Alternatively, when NECs form in the spinal cord, they undergo a period of expansion through cell division before initiating neurogenesis. This period of quiescent neurogenesis is achieved by uniform, elevated expression of the bHLH repressors such as Hes-1, which blocks proneural gene activity. In this case, the uniform expression of Hes-1 appears to occur by blocking their negative feedback loop with the Id proteins. Finally, when neurogenesis begins, the expression of the repressor bHLH proteins becomes dynamic, oscillating in NECs as a consequence of negative feedback on their own expression, and positive regulation by the Notch signaling pathway during a patterning process called lateral inhibition. As discussed in more detail below, this dynamic repression within the NECs limits the extent of neurogenesis and maintains undifferentiated progenitors, while allowing other progenitors to differentiate. A major challenge, therefore, is to determine how the patterning genes, and other mechanisms, control the expression and activity of bHLH repressors and Ids, thus controlling the location, timing, and extent of neurogenesis.

One of the important positive inputs into the expression of the bHLH repressors involves local cell–cell interactions mediated by the Notch pathway. The enhancers for these genes are directly regulated by the Notch signal transduction pathway via binding sites for a DNA binding protein, referred to here as Suppressor of Hairless (Su(H)). In the absence of Notch signaling, Su(H) acts as a transcriptional repressor that is thought to actively inhibit the expression of the bHLH repressors. However, upon Notch receptor activation by ligand binding, the Notch intracellular domain (ICD) is released from the membrane, moves to the nucleus, and converts Su(H) from a repressor into an activator, thus rapidly inducing gene expression. As a consequence, activating the Notch pathway induces the expression of repressor bHLH genes and thereby inhibits neurogenesis, while inhibiting the Notch pathway enhances the levels of neuronal differentiation within a pool of neural precursors. Significantly, the proneural proteins can potently activate the expression of Notch ligands, such as those related to *Drosophila* Delta. Thus, proneural proteins not only promote neuronal differentiation cell autonomously, but by activating Delta they inhibit neuronal differentiation in their neighbor's non-cell autonomously. This interaction of the proneural proteins with the Notch pathway underlies the process of lateral inhibition, and appears to be a critical factor in maintaining a fraction of the NECs as undifferentiated progenitors during neurogenesis. In addition, Notch signaling not only maintains undifferentiated progenitors, but in the right context, it can also activate pathways involved in astroglia formation. As such, the Notch pathway has become a powerful tool that can be used to regulate the timing and extent of both neuro- and gliogenesis by neural progenitors.

Accordingly, one can imagine scenarios where patterning genes act by targeting the activity of the Notch pathway, perhaps by targeting a number of proteins known to be Notch modulators. Activity of the Notch receptor, for example, is modulated by post-translational modification mediated by glycosyl-transferases encoded by the vertebrate homologs of the *Drosophila* Fringe gene. The Fringe homologs are dynamically expressed within neural precursor populations, where they may influence the activity of the Notch pathway. A number of mechanisms have been proposed to change the half-life of the activated Notch receptor, ICD, thus altering the efficacy of Notch activity. Finally, another potential mechanism of modulating Notch activity is by changing the activity and/or expression of the ligands. In this respect, an important factor in ligand activity appears to be their clustering and endocytosis via ubiquitination by specific E3 ligases. In sum, modulation of Notch activity is likely to be one way

in which the output of the patterning genes could target the activity of the proneural proteins during neurogenesis.

Divergence of Proneural Target Genes During Neuronal Differentiation

The activation of the bHLH cascade results in a burst of proneural activity in NECs that apparently drive the changes associated with neuronal differentiation. However, as these changes occur, proneural gene expression is lost, suggesting that the bHLH cascade is not required after the initial stages of neuronal differentiation because it activates a variety of downstream targets that carry out different aspects of neuronal differentiation, such as cell cycle exit, up-regulation of neuronal gene expression, neuronal migration, neuron cell type specification, etc. Since some of these targets also appear to interact with the proneural cascade, the following discussion will highlight some of critical targets of the bHLH cascade during the phase of neuronal differentiation.

Regulation of Neuronal Differentiation by the Sox Proteins

As described above, the proneural proteins induce the differentiation of progenitors into neurons by activating gene expression required in neurons, while down-regulating those in precursors. One way this appears to be achieved is by interactions with members of the Sox superfamily. When NECs form during neural induction, they express members of the SoxB1 subfamily, Sox1-3, which maintain the expression of progenitor genes, but also inhibit the activity of the proneural proteins. Proneural proteins appear to overcome this block in two ways: (1) by inducing the expression of Sox21, a member of the SoxB2 family that antagonizes the activity of Sox1-3; and (2) by inducing the expression of Sox4 and Sox11, members of the SoxC family that transcriptionally activate the expression of pan-neuronal genes perhaps directly. Significantly, the proneural-driven switch in SoxC activity does not drive cell cycle exit, but rather a subroutine that turns off genes whose expression is required by progenitors while turning on that required by neurons.

REGULATION OF NEURONAL DIFFERENTIATION BY REST/NRSF

Proneural proteins may also activate the expression of neuronal genes by targeting a repressor, called REST/NRSF, which plays an important role in keeping many neuronal genes in an off state, both in non-neuronal cells, as well as in neural precursors. REST/NRSF can occupy binding sites that are present in the enhancer of many neuronal genes, thereby recruiting a slew of modifying enzymes that maintain chromatin in a repressed state. REST/NRSF can also repress the expression of Sox4 and Sox11, which, as discussed above, are activated by the proneural proteins during neurogenesis and are required for neuronal gene expression. During neuronal differentiation, therefore, an important step is to relieve the repressive chromatin modifications that are set in place by REST/NRSF. This may occur by altering the activity of REST/NRSF with small RNAs, or by simply removing REST/NRSF by targeting it to the proteosome-degradation pathway by the E3 ligase, SCFβ-TrCP. Both mechanisms may be set in place by proneural activity, although how this occurs is currently unknown.

SWITCH FROM NEUROGENESIS TO GLIOGENESIS

In the discussion above, we have outlined how neural progenitors arise in the embryo, and how patterning of these progenitors engages a proneural cascade that drives neurogenesis at the right time and place. However, one hallmark of the developing vertebrate CNS is that once neurogenesis is complete, the NECs then form the progenitors that give rise to the myelinating oligodendrocytes and astrocytes. Each of these neural cell types does not appear to have a dedicated progenitor that forms when NECs are first established. Instead, the initial patterning of NECs seems to establish conditions that promote neurogenesis, but as time goes by, the NECs that have not differentiated are repatterned, establishing conditions that suppress neurogenesis, while promoting gliogenesis. In the ventral spinal cord, for example, the progenitor domain that gives rise to motor neurons is initially established during D–V patterning by activating the restricted expression of Olig2, as described above. Once the production of motor neurons ceases, the same pool of progenitors become a major source of oligodendrocyte precursors (OPCs), and even later can give rise to astrocytes and the ependyma. Significantly, as these progenitors stop neurogenesis, they then use the same factors that promote neurogenesis, such as Olig2 and Mash1, to give rise to OPCs. Thus, factors that promote neurogenesis in one context, promote oligodendrocyte formation in another. The implication is that a switch occurs in NECs, through the temporal change in the expression of genes that modulate the way in which Olig2 and Mash1 promote differentiation within NECs. For example, one model is that temporal changes in the expression of such transcription factors as Sox9 or NF1A within NECs preclude neurogenesis, while promoting conditions required for gliogenesis.

SUMMARY

The development of the CNS can be represented as a series of fate choices that are progressively made by embryonic

cells in response to both intrinsic and extrinsic cues. One of the first fate decisions is made in the ectoderm, where cells form the NECs of the neural plate rather than differentiating into nonneural tissues. This choice apparently can occur by default, suggesting that embryonic cells can form NECs in the absence of extrinsic instructions. However, a key process during neural induction is neural patterning, during which a complex network of gene expression is established along the neuraxis, thereby specifying the position and subsequent fate of NECs. These complex genetic networks, many of which involve homeodomain transcription factors, dictate patterns of neurogenesis by controlling when and where NECs undergo neuronal differentiation. Significantly, the patterning genes appear to regulate neurogenesis by converging on the activity of the proneural bHLH proteins, which function as molecular switches to initiate neuronal differentiation by promoting cell cycle arrest, expression of neuronal differentiation genes, suppression of glial differentiation genes, and the activation of neuronal subtype genes. Thus, the neural precursors for the CNS initially choose their fate by default, but neural patterning is instrumental in instructing their subsequent neuronal fate by establishing a complex code of gene expression that drives the bHLH cascade at the proper time and place. The remaining challenge is to determine how the bHLH cascade is engaged in a myriad of ways to produce the appropriate number and types of neurons that comprise each region of the CNS along the neuraxes.

KEY WORDS

Neural induction A key event that occurs during vertebrate development when a region of the early embryo acquires the ability to form neural tissue. Transplant experiments in the early 1900s showed that the acquisition of a neural fate requires inductive signals, whose molecular identities have only recently been elucidated.

Neural patterning The events that specify the fate of neural progenitors along the anterior–posterior and dorsal–ventral axis of the nervous system. These events typically involve morphogens that are produced within specialized structures called organizing centers, and act in a concentration-dependent fashion on neighboring regions of neural progenitors to dictate fate.

Neurogenesis The events that drive the differentiation of neurons from neural progenitors, including withdrawal from the cell cycle, detachment from a neuroepithelium, migration to a particular location within neural tissue, and the elaboration of axonal and dendritic projections.

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Chapter 19

The Nervous System

Lorenz Studer

INTRODUCTION

Over the last few years embryonic stem (ES) cells have emerged as a powerful tool to study brain development and function. The recent isolation of human ES (hES) cells has stimulated ES cell research directed towards therapeutic cell applications. The central nervous system (CNS) has been proposed as one of the prime targets for ES cell therapies, due to early successes in directing ES cell fate towards neural lineages, the experience with fetal tissue transplantation, and the devastating nature of many CNS diseases with very limited treatment options. Some of the most striking advantages of ES cells compared with any other cell type are: extensive self-renewal capacity and differentiation potential; access to the earliest stages of neural development; and ease of inducing stable genetic manipulations.

NEURAL DEVELOPMENT

The neural plate is derived from the dorsal ectoderm and induced by "organizer" signals derived from the underlying notochord. The dominant model of neural induction is the default hypothesis. This hypothesis states that neural tissue is formed spontaneously in the absence of BMP signaling during early gastrulation, while exposure to BMP signals causes epidermal differentiation. Accordingly, signals emanating from the organizer essential for neural induction are BMP inhibitors such as chordin, noggin, follistatin, and cerberus. However, FGF signals emanating from precursors of the organizer prior to gastrulation have been implicated in providing a "prepattern" for neural induction via activation of Sox3 and ERNI. Other players during neural induction are IGF and Wnt signals.

After the formation of the neural plate cells undergo a well-defined set of morphological and molecular changes leading to the formation of neural folds and neural tube closure. This is followed by orchestrated waves of neural proliferation and differentiation. Of particular importance in determining specific neural fates are signals that provide regional identity, both in the antero–posterior (A–P) and dorso–ventral (D–V) axis, and define domains of distinct expression of homeodomain proteins and bHLH transcription factors. The leading hypothesis of A–P axis specification states that anterior fates are default during early neural induction, while FGF, Wnt, and retinoid signals actively posteriorize cell fates. D–V identity is determined by the antagonistic action of Sonic hedgehog (Shh) secreted ventrally from the notochord and floor plate, and BMPs and Wnt signals dorsally. There is ample evidence from explant and ES cell differentiation studies that confirm a concentration-dependent role of Shh to define specific progenitor domains within the neural tube. A role for BMPs in dorsal neural patterning has been suggested from explant and ES cell differentiation studies. Gain of function studies in mice overexpressing the BMP receptors under control of the regulatory elements of the nestin gene are pointing to a role for BMPs in dorsal patterning. However, loss-of-function studies of the BMP receptors suggest a much more limited role for BMPs in dorsal patterning, affecting primarily choroids plexus development. Dorsal patterning factors also include additional TGFβ-family members such as GDF and activin and Wnts, known to be critical in the establishment of the neural crest.

Subsequent differentiation of patterned neural precursor cells occurs in a stereotypic fashion, with neurons being born first followed by astroglial and oligodendroglial differentiation. Onset of neuronal differentiation is controlled via inhibition of the Notch pathway that represses proneural bHLH genes. Astrocytic fate is established via activation of Jak/Stat signals, which exert an instructive role on multipotent neural progenitors to drive astrocytic differentiation. However, recent findings on the neurogenic properties of radial glial, and the identification of adult neural stem cells as a cell expressing astrocytic markers, suggest a more complex and dynamic interaction between neural stem cell and astrocytic fates. Oligodendrocytes were

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believed to derive from bi-potent glial precursor, termed O2A progenitors, or other glial restricted precursors. However, there is also data demonstrating a lineage relationship between oligodendrocytes and ventral neuron types such as motoneurons in the spinal cord and forebrain interneurons by their shared requirement for Olig2 expression. Furthermore, *in vivo* lineage studies have shown that SVZ stem cell populations can also contribute to the generation of oligodendroglial progeny. While a detailed discussion of the signals directing specific neuron fates is beyond the scope of this chapter, a few examples will be provided in the section on Derivation of ES-derived Neurons.

NEURAL STEM CELLS

Neural stem cells have been isolated both from the developing and adult brain. Nearly 20 years of intensive research have demonstrated self-renewal of neural stem cells and multi-lineage differentiation into neurons, astrocytes, and oligodendrocytes. However, in most *in vitro* and *in vivo* paradigms, neural stem cells do not give rise to all the neuron types present in the adult brain, but are limited largely to the production of GABA and glutamate neurons. Experiments that marked adult SVZ stem cell populations in different regional subdomains showed strong evidence for region-specific bias in the production of specific neuron types such as Pax6+/TH+ cells or Calretinin+/GABA+ cells. These biases in neuron subtype differentiation potential appear to reflect cell autonomous properties of the SVZ stem cell compartment, and are retained even upon extensive *in vitro* culture.

The isolation and propagation of neural stem cells *in vitro* can be achieved by selective growth and proliferation conditions. The most commonly used method is the neurosphere assay. Under these conditions, neural precursors are grown as free-floating spheres in the presence of EGF and FGF-2. Human neurosphere cultures are often also supplemented with LIF. Neurospheres in rodents can be formed from single cells, and the capacity for neurosphere formation is often used as an assay to demonstrate stem cell properties. For example, the prospective isolation of neural stem cells via surface marker expression (AC133, Lex1, or combinations of surface markers), was based on the ability of these cells to form neurospheres *in vitro*. These data need to be interpreted cautiously, as neurosphere formation is not necessarily a true test of stem cell identity, and neurospheres can be formed efficiently from cells that exhibit transit amplifying properties *in vivo*. Furthermore, neurospheres contain many differentiated cells in addition to the progenitor/stem cell population.

An alternative to neurospheres are monolayer culture techniques, where neural precursor/stem cells are grown on an attachment matrix, such as fibronectin or laminin, in the presence of FGF2. These conditions are more amenable to study the precise lineage relationship of individual cells, and complete cell lineage trees have been worked out under such conditions. A recent variation of the monolayer culture technique is the NS culture system, based on the proliferation of radial glia-like NSC progeny in the presence of FGF2 and EGF. NS cultures are thought to exhibit improved maintenance of neurogenic fate potential following long-term culture, and may represent one of the few examples of a symmetrically-dividing stem cell population. However, one of the remaining important limitations of current neural stem cell technology is the limited control over neural patterning and neuronal subtype specification. The derivation of midbrain dopamine neurons has served as a model for these problems. Functional midbrain dopamine neurons can be derived from short-term expanded precursor cells isolated from the early rodent and human midbrain. However, long-term expansion causes a dramatic loss in the efficiency of midbrain dopamine neuron generation. Several strategies have been developed in an attempt to overcome these problems, ranging from exposure to complex growth factor cocktails changing oxygen levels to the transgenic expression of Nurr1, a key transcription factor during midbrain dopamine neuron development. However, none of the approaches has succeeded in deriving fully-functional midbrain dopamine neurons from long-term expanded neural stem cells. The existence of a defined neural stem cell compartment in the adult with a very broad neuron subtype differentiation potential has remained elusive. However, recent studies suggest that such cells can be isolated during the neural differentiation of ES cells, and may represent a very early stage neuroepithelial stem cell compartment.

NEURAL DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

Neural Induction

There are at least three main strategies for the neural induction of ES cell *in vitro*: embryoid-body (EB)-based systems; stromal feeder mediated neural induction; and protocols based on default differentiation into neural fates (Figure 19-1).

Embryoid-body Based Protocols

EBs are formed on aggregation of ES cells in suspension culture. The interactions of cells within the EB causes cell differentiation, mimicking early embryonic development including the induction of early extraembryonic endoderm and various aspects of gastrulation. EBs represent a useful assay to generate derivatives of all three germ layers *in vitro*. Various modifications of the basic protocol have been developed to drive neural induction, and to select and expand EB-derived neural precursors.

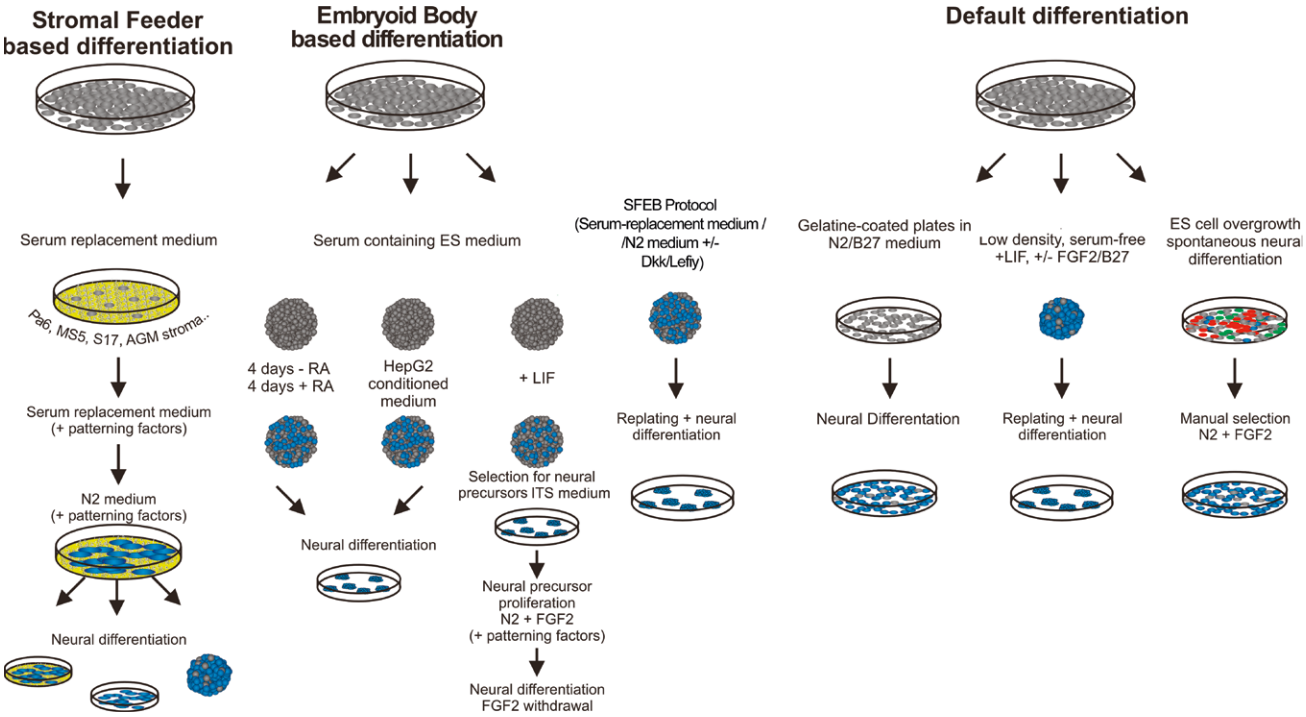


FIGURE 19-1 Basic techniques for inducing neural differentiation in embryonic stem cells *in vitro*. Embryoid-body based protocols (left panels) are initiated by the aggregation of undifferentiated ES cells. Neural differentiation (represented in blue) is promoted either by exposure to RA, HepG2 (hepatocarcinoma cell line) conditioned medium or via neural selection in defined medial. Classic studies are cited (in green) for each of the three main EB based strategies. Stomal feeder mediated differentiation is obtained upon plating undifferentiated ES cells at low density on stromal feeder cell lines derived from the bone marrow of the AGM region of the embryo. Serum-free conditions are required throughout the protocol. Conditions can be readily adapted to achieve neural subtype-specific differentiation for a large number of CNS cell types. Classic studies are cited (in green) PA6 and MS5 mediated differentiation. Default differentiation. Neural differentiation is achieved by reducing endogenous BMP signals via plating cells at low density under minimal medium conditions, or in the presence of the BMP antagonist noggin. Neurally-committed cells can also be mechanically isolated and propagated from plates exhibiting spontaneous neural differentiation after overgrowth of ES cells. The SFEB (serum-free embryoid body) protocol combines aspects of EB formation with the default induction strategy.

The first EB-mediated neural differentiation protocol was based on exposure to retinoic acid (RA) for four days following four days of EB formation in the absence of RA (so called 4−/4+ protocol). In addition to neural induction, RA treatment also exhibits a strong caudalizing effect on A–P patterning mediated through activating the Hox gene cascade. An alternative EB-based strategy is exposure to conditioned medium derived from a hepatocarcinoma cell line (HepG2), which appears to induce neuroectodermal fate directly. Accordingly, HepG2-treated aggregates do not express endodermal or mesodermal markers, but appear to give rise directly to neural progeny. The active component within HepG2-conditioned medium is not known, although data suggest that at least two separable components are responsible for this activity. A third EB-based strategy makes use of neural-selective growth conditions. EB progeny is kept under minimal growth conditions in serum-free medium containing insulin, transferrrin and selenite (ITS medium). Under these conditions most EB-derived cells die and a distinct population of immature cells emerges that expresses increasing levels of the intermediate filament nestin. These nestin+ precursors can be replated and directed towards various neuronal and glial fates using a combination of patterning, survival and lineage promoting factors (see the sections on Derivation of ES-derived Neurons and ES-derived Glia). The most recent variation of an EB-like differentiation protocol is the so-called SFEB (serum-free EB) protocol. The SFEB approach is distinct from other EB-based strategies, as it does not involve a gastrulation-equivalent intermediate stage but directly yields neural progeny in the absence of serum or other inducing signals. Therefore, SFEB-based protocols combine ESC aggregation with the default neural induction concept discussed below. SFEB-based protocols are particularly attractive for generating anterior neural fates, such as cortical or ventral forebrain fates. However, variations of this approach can be adapted to the derivation of many neuron types, including spinal motoneurons.

Stromal Feeder Mediated Neural Induction

Bone marrow-derived stromal cell lines have been used for many years to support the growth of undifferentiated hematopoietic stem cells. Over the last 10 years, it has

become evident that several stromal cell lines also exhibit neural-inducing properties in co-culture with mouse ES cells. Stromal cell lines with the highest efficiencies of neural induction are typically at the preadipocytic stage of differentiation and are isolated from the bone marrow (e.g., PA-6, MS5, S17) or the aorta-gonad-mesonephros (AGM) region. The molecular nature of this stromal-derived inducing activity remains unknown. However, the efficiency and robustness of neural induction using stromal feeder approaches is high compared with alternative protocols, and differentiation can be directed towards specific regional fates in response to extrinsic factors, despite the fact that these protocols have been particularly useful for the derivation of midbrain dopamine neurons from ESCs.

Neural Differentiation by Default

Co-culture-free direct neural differentiation protocols are based on the default hypothesis that absence of signals in primitive ectodermal cells will lead to neural differentiation. Several neural induction approaches in mouse and human ES cells have been developed based on this approach. In addition to the SFEB protocol discussed above, the original default strategy using a cell aggregation approach was based on ultra-low density plating. Under these conditions, rapid expression of neural markers is observed in the few surviving colonies. However, the cells expressing neural markers were reported to represent a "primitive" neural precursor population, distinct from fully committed neural progeny. An even more promising strategy may be the direct induction of neural fates in the absence of any aggregation or stromal feeder co-culture. Direct adherent monolayer culture conditions have been developed for neural induction of mouse and human ES cells in the presence of N2 medium supplemented with B27 and FGFG2. However, these protocols need to be validated critically, addressing the contribution of neural induction versus selective survival of spontaneously differentiating neural progeny, given the relatively low overall yield. Most recent work in human ES cells suggests that adherent monolayer cultures can be developed that are extremely efficient at driving neural induction under simple serum-free conditions in the presence of inhibitors that block both the BMP and TGFβ/Activin branch of SMAD signaling.

Derivation of Embryonic Stem Cell-derived Neurons

Spontaneous differentiation into neurons occurs rapidly on neural induction of mouse ES cells. Neuronal subtype specification can be influenced by the mode of neuronal induction. This is particularly the case for RA induction protocols. The fundamental strategy for achieving neuronal subtype specification is based on mimicking patterning events that define A–P and D–V patterning events *in vivo*.

Midbrain Dopaminergic Neurons

Derivation of midbrain dopamine neurons from ES cells has been of particular interest, due to the clinical potential for dopamine neuron transplants in Parkinson's disease. Protocols for the dopaminergic differentiation of mouse ES cells are based on studies in explants that identified FGF8 and Shh as critical factors in midbrain dopamine neuron specification. The effect of Shh/FGF8 on ES-derived neural precursors was first described using an EB-based five-step differentiation protocol. Under these conditions, up to 34% of all neurons expressed tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine. A further increase in dopamine neuron yield (nearly 80% of all neurons expressing TH) was obtained in Nurr1 overexpressing ES cells. Midbrain dopaminergic differentiation was also obtained using co-culture of ES cells on the stromal feeder cell line (PA6) with 16% of all neurons expressing TH in the absence of Shh and FGF8. These results were initially interpreted as PA6 exhibiting a specific patterning action that promotes dopamine neuron fate. However, later studies demonstrated that neural precursors induced on stromal feeders can be shifted in A–P and D–V identity, and reach a yield of up to 50% of all neurons expressing TH without a need for transgenic Nurr1 expression. Numbers of TH neurons need to be interpreted carefully in all *in vitro* differentiation studies, as TH is an unreliable marker for identifying midbrain dopamine neuron fate. TH is expressed in other catecholaminergic neuron types, such as noradrenergic and adrenergic cells, and can be induced in many cell types under various unspecific external stimuli, such as stress or hypoxia. It is, therefore, essential to use additional markers to confirm dopamine neuron identity, and to perform functional studies *in vitro* and *in vivo*. Another key advance in generating highly-enriched populations of midbrain dopamine neurons from mouse ES cells is the transgenic expression of Lmx1A, under control of a neural precursor specific promoter. Lmx1A is thought to act as a cell fate determinant within the ventral midbrain, and greatly promotes the generation of authentic midbrain dopamine neurons from mouse ESCs differentiated in the presence of Shh/FGF8. The most recent studies stress the importance of another key transcription factor, FoxA2, in midbrain dopamine neuron development, and point to the floor plate origin of midbrain dopamine neurons. Such novel developmental insights have spurred attempts to generate ES cell-derived midbrain dopamine neurons via a defined floor plate intermediate.

Serotonergic Neurons

The developmental origin of serotonergic neurons is closely related to that of midbrain dopamine neurons. Both neuronal subtypes are dependent on signals emanating from the

isthmic organizer. Accordingly, serotonergic neurons are a major "contaminating" neuronal subtype in protocols aimed at the derivation of midbrain dopaminergic cells. Application of FGF4, preceding FGF8 and Shh application ectopically, induces serotonergic neurons in explant and has been reported to enhance serotonergic differentiation in EB and stromal feeder-based neural induction protocols. Novel strategies under development to refine serotonergic differentiation from ES cells are based on the developmental studies on the role of the transcription factor Lmx1b and Nkx2.2 during serotonergic differentiation.

Motor Neurons

Development of spinal motor neurons has been studied in great detail, using lineage tracing and a variety of loss and gain of function approaches. Early studies demonstrated that cells expressing markers of motoneurons can be generated using an EB induction protocol in combination with RA exposure (2−/7+). More systematic approaches have defined the critical role for combining RA exposure with Shh treatment for generating ES-derived motoneurons at very high efficiencies. These studies provided a classic example of how developmental pathways can be harnessed to direct ES cell fate *in vitro*. The use of an ES cell line expressing GFP under the control of the HB9 promoter allowed simple identification and purification of ES-derived motoneurons. The *in vivo* properties of these cells were demonstrated by contribution to the motoneuron pool after transplantation into the developing chick spinal cord. Efficient derivation of motoneurons has also been achieved using stromal feeder protocols in combination with Shh and RA treatment. The derivation of somatic motoneurons has been demonstrated from many pluripotent sources, including human-induced pluripotent stem cells for modeling motoneuron disease. Current challenges for *in vitro* motoneuron differentiation protocols are the selective generation of specific motoneurons with distinct A–P and columnar identity. Other important factors for clinical translation in the treatment of amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and spinal cord injury (SCI) include strategies to modulate motoneuron survival, axonal outgrowth, target selection, and specificity of muscle innervation. In the context of ALS, there has been considerable evidence of a strong non-cell autonomous component contributing to the disease phenotype. Despite these challenges, the ability to generate somatic motoneurons at large quantity and purity remains one of the best examples demonstrating the success of directed neural differentiation protocols in pluripotent stem cells.

GABA Neurons

GABA cells are the main inhibitory neuron type within the brain, and are the dominant neuron type in the basal forebrain, particularly in the striatum. The presence of GABAergic neurons during ES cell differentiation *in vitro* has been reported under various conditions, including the classic 4−/4+ EB-based differentiation protocols, which yield approximately 25% GABA neurons. The presence of GABA neurons has also been reported under default neural induction conditions. Directed differentiation to GABA neurons has been achieved using a stromal feeder-based approach. Neural induction on MS5 is followed by neural precursor proliferation in FGF2, and subsequent exposure to Shh, FGF8. The delayed application of FGF8 and Shh promotes ventral forebrain identities, as determined by the expression of the forebrain specific marker FOXG1B (BF-1) and the increase in GABAergic differentiation. SFEB-based neural induction protocols have been particularly useful in deriving neural cells enriched for forebrain fates including GABA neurons. Efforts are ongoing to derive highly-purified sub-populations of GABA neurons, such as cortical interneurons or medium spiny striatal neuron types critical in the context of epilepsy, Huntington's disease, or stroke.

Glutamate Neurons

Glutamate neurons can be readily obtained at high efficiencies from mouse ES cells. For example, in the original EB protocols, approximately 70% of all neurons were glutamatergic and ES-derived neurons, with NMDA and non-NMDA receptor subtypes have been described. Very similar neuron subtype compositions have been obtained with various related protocols. More detailed physiological data on glutamatergic neurons have been reported after co-culture of ES-derived neurons on hippocampal brain slices. Interestingly, this study suggested a possible bias towards establishing AMPA versus NMDA-type synaptic contacts. Most recent protocols have yielded ES-derived precursors biased towards cortical fates on pharmacological inhibition of Shh signaling, using a single one-step culture technique. Interestingly, tracing the lineage potential of such ES cell-derived neural precursors demonstrates considerable self-organization potential, and allows the clonal recreation *in vitro* of temporal neuronal subtype diversity observed during cortical development.

Other Neuronal and Neural Subtypes

While the presence of about 5% glycinergic neurons has been reported using the classic 4−/4+ EB protocol, no directed differentiation protocols have been developed for the derivation of this neuron type. Another interesting neuron types generated from ES cells are hypothalamic neurons that are generated by minimizing extrinsic signaling conditions. Finally, there are also emerging strategies for generating placode-derived neural cell types, including otic precursors, from ES cells. ES cell-derived otic precursors

were shown to engraft and differentiate into cells expressing markers of mature hair cells *in vivo*.

Neural Crest Differentiation

The neural crest is a transient structure formed from the most dorsal aspects of the neural tube of the vertebrate embryo. It contains migratory cells that form the peripheral nervous system, including sensory, sympathetic, and enteric ganglia, large parts of the facial skeleton and various other cell types including Schwann cells, melanocytes, and adrenomedullary cells. ES cells provide a powerful assay to study neural crest development *in vitro*. The main strategy for deriving neural crest-like structures from ES cells is based on BMP exposure, alone or in combination with Wnt and FGF signals, during early neural induction stages. This can be achieved in both mouse and human ES cell systems, with the development of sensory and sympathetic neurons in a BMP dose-dependent manner. More recent studies in human ES cells have defined conditions for neural crest precursor cell induction, and prospective isolation of neural crest stem cell populations. Human ES cell-derived neural crest stem cells can be further directed towards multiple neuronal, glial, and mesenchymal fates exhibiting features of cranial neural crest precursors. Finally, human ES cell-derived neural crest precursors are capable of *in vivo* engraftment in the developing chick embryo, and show migratory and differentiation behaviors compatible with neural crest identity. Such studies clearly indicate that the directed differentiation and prospective isolation of various neural crest populations is feasible, and provides an unlimited source of human ES cell-derived neural crest precursors for basic developmental studies, disease modeling, and regenerative medicine.

Embryonic Stem Cell-derived Glia

Neural progenitors derived from mouse ES cells can be readily differentiated into astrocytic and oligodendroglial progeny under conditions similar to those described from primary neural precursors. The first reports on the glial differentiation of mouse ES cells were based on the 4−/4+ EB protocols or multi-step EB differentiation protocols. However, studies have demonstrated the derivation of glial cells under various neural induction conditions, and protocols have been published for the selective generation of astrocytes and oligodendrocytes from ES cell-derived neural precursors.

Oligodendrocytes

Highly-efficient differentiation into oligodendrocytes was first reported using a modified multi-step EB-based protocol. ES-derived neural precursors were expanded with FGF2, followed by FGF2 + EGF, and FGF2 + PDGF. These conditions yielded a population of A2B5+ glial precursors that are capable of differentiation into both astrocytic (~36% GFAP+) and oligodendrocytic (~38% O4+) progeny upon mitogen withdrawal. The 4−/4+ EB RA induction protocol has been optimized for the production of oligodendrocytic progeny, in combination with genetic selection strategies. Oligodendrocytic differentiation is achieved in RA-induced EBs after expansion in FGF2, and followed by dissociation and replating in serum-free medium containing FGF2 and Shh. The final step involved Shh and FGF2 withdrawal, and the addition of PDGF and thryoidhormone (T3). Under these conditions, ~50% of all cells express oligodendroglial markers. Stromal feeder-mediated induction, initially thought to bias towards neuronal progeny, can also be readily adapted to derive oligodendrocytes at very high efficiencies. In human ES cells there have been reports claiming the derivation of pure oligodendrocyte populations without the need for cell selection. However, there remains controversy regarding the protocols required for inducing authentic oligodendrocytes from human ES cells, and the exact nature and developmental stage of the cells expressing oligodendrocyte markers in different protocols.

Astrocytes

Highly-efficient differentiation of ES cells into astrocytes has been reported using stromal feeder-mediated neural induction followed by sequential exposure to FGF2, bFGF/EGF, EGF/CNTF, and CNTF. Over 90% of all cells expressed the astrocytic marker GFAP under these conditions. Significant numbers of GFAP cells were also obtained using HepG2-mediated neural differentiation or multi-step EB protocols. Glial progenitors obtained with a multi-step EB protocol were recently "transplanted" *in vitro* into hippocampal slices, and revealed that full physiological maturation of ES-derived astrocytes can be achieved on interaction with an appropriate host environment. The efficient derivation of astrocytes and oligodendrocytes from human ES cells is associated with extensive *in vitro* culture, suggesting the need for additional factors controlling gliogenic competency in these cells.

Lineage Selection

Lineage selection based on surface markers or the cell type-specific expression of promoter-driven selectable markers in ES cell progeny provides a complementary approach to directed differentiation protocols. The use of genetic markers is particularly attractive in ES cells, due to the ease of inducing stable genetic modifications and the availability of large libraries of transgenic and gene-targeted mice and ES cells. A classic example of using a

genetic reporter for the isolation of neural progeny from mouse ES cells *in vitro* is the *Sox1::EGFP* knockin cell line. Other classic examples of ES lines used successfully for the genetic identification and purification of neural precursors and neuronal and glial progeny *in vitro* include the *tau::eGFP* knockin cells (marking neuronal progeny), GFAP transgenic reporter cell (marking astrocytes), *GAD67::lacZ*, HB9:eGFP, or Pitx3::eGFP reporter lines for the isolation of various neuron subtypes.

Systematic recent efforts to generate ES cell reporter lines are based on the use of bacterial artificial chromosome (BAC) transgenic reporters. BACs can be obtained for any gene, can be readily manipulated to carry a reporter of interest, and are stably introduced into mouse or human ES cells. This approach is inspired by the large-scale use of BAC transgenic mice, where many hundreds of transgenic mice strains have been generated marking specific neural lineages (www.gensat.org). The use of the technology directly in ES cells offers to extend this effort to human ES cells, and to the development of comprehensive ES cell libraries for basic and translational studies. Examples of BAC transgenic neural reporter lines already available in human ES cells include *Hes5::GFP* (marking neural precursors), *Dll1::eGFP* (marking neuroblasts), and *HB9::eGFP* (marking somatic motoneurons).

NEURAL DIFFERENTIATION OF HUMAN AND NONHUMAN PRIMATE ES CELLS

While many of the neural differentiation protocols developed in mouse ES cells can be adapted to primate and human ES cell systems, there are a number of important differences between mouse and primate protocols. First, the time periods required for neural induction and neuronal and glial subtype specification in human ES cell progeny are very protracted. Second, while transcriptional control of pluripotency is conserved between mouse and human cells, the extrinsic signaling required for maintaining self-renewal of the pluripotent state is highly distinct. Accordingly, the signals required for inducing loss of pluripotency and adopting neural commitment are distinct. For example, inhibition of activin/TGFβ signaling greatly enhances neural induction in human, but not mouse, ES cells. Third, neural induction in human ES cells is generally associated with neural rosette formation, which is less commonly observed in mouse ES cells.

The first efficient protocols for the neural differentiation of human ES cells were based on a modified multistep EB approach. ES cells are aggregated for a short time (~4 days), and subsequently replated under serum-free conditions in the presence of FGF-2. Under these conditions, neural precursors can be readily-identified, based on the formation of multilayered epithelia, which are termed rosettes. These neural precursor cells can be enzymatically separated from the surrounding cell types, and grown to purity under neurosphere-like conditions in the presence of FGF-2, yielding neurons and astrocytes upon differentiation. The main neuronal subtypes are GABAergic and glutamatergic phenotypes. A similar spectrum of differentiated progeny was obtained when hES-derived neural precursors were obtained after spontaneous neural differentiation by overgrowth of undifferentiated cells. Rosette-like structures were manually isolated under microscopic view, and subsequently grown and passaged under neurosphere-like conditions. A third strategy that yielded differentiated cell populations enriched for neural precursors was based on a modified EB RA induction protocol, followed by lineage selection in attached cultures using serum-free conditions and supplementation with bFGF.

A number of protocols have subsequently been developed that yield better control over neuronal subtype specification for the derivation of midbrain dopamine neurons, somatic motoneurons, and GABA ergic forebrain neuron types. Interestingly, PA6-mediated neural induction caused differentiation into retinal pigment epithelial cells, in addition to neural differentiation. Human ES cells can be readily coaxed into neuronal populations with 70% of the neurons expressing TH. The first protocols achieving such high efficiencies were based on the neural induction of human ES cells on MS5 stroma, followed by sequential exposure to Shh/FGF8 and differentiation in the presence of AA, BDNF, and GDNF. However, EB-based strategies have been adapted to yield comparable efficiencies in DA neuron yield. One important question is the need for additional inducing signals, such as those provided by midbrain specific glia or via overexpression of key transcription factor such as Lmx1A or FoxA2, and the question of whether a floor plate intermediate is a requirement for midbrain dopamine neuron generation from human ES cells, as suggested by studies in the developing mouse embryo. Similar to the studies in mouse ES cells, SFEB-based protocols have been developed for human ES cell differentiation that have been particularly useful for generating anterior neural fates. In human ES cells, SFEB formation is greatly enhanced when treating cells with the ROCK inhibitor Y-27632 during dissociation, and early culture increased viability and SFEB formation in human ES cell progeny. Studies in human ES cells have further characterized neural rosettes, and identified rosette neural stem cell type (R-NSCs) capable of self-renewal while retaining a very broad differentiation potential along lineages of both the central and peripheral nervous system. These findings indicate that R-NSCs may represent a rare example of a neural stem cell population that appears to retain the potential to respond to patterning factors that define A–P and D–V identity and are capable of recreating the full neuronal subtype diversity.

DEVELOPMENTAL PERSPECTIVE

Highly reproducible *in vitro* differentiation protocols, and an increasing number of available ES cell reporter lines, provide powerful tools for establishing cell-based developmental screens. In addition, large-scale gene trapping approaches have yielded individual ES cells lines mutant for a large proportion of the mouse genome (e.g., http://www.genetrap.org). Several ES cell-based *in vitro* differentiation screens have been carried out to assay for genes involved in neural/neuronal induction. Such screens have identified a number of molecules impacting mouse ES cell self-renewal and neural differentiation, including several compounds affecting Wnt signaling. Recently, it also has become possible to perform HTS assays in human ES cells, after careful optimization of plating and dissociation techniques. Such screens have identified a number of additional molecules affecting human ES cell self-renewal and differentiation, including several FDA approved drugs. The availability of high-throughput functional genomic approaches, such as genome-wide RNAi inference and large-scale cDNA libraries, will further extend the power of ES cell biology to systematically probe normal development and disease *in vitro*. One long-term goal of such studies could be the establishment of a high-resolution functional lineage map of the nervous system in both mouse and human ES cells.

THERAPEUTIC PERSPECTIVES

One of the driving forces behind deciphering the developmental program that controls cell fate specification is the hope that such insights could be harnessed for generating specialized cells for therapy. However, despite the excitement about the potential of ES cells in neural repair, there are only few examples where such approaches have been tested in animal models of disease.

Parkinson's Disease

One of the most widely-discussed applications is the derivation of unlimited numbers of dopamine neurons from human ES cells for the treatment of Parkinson's disease (PD). PD is particularly attractive for cell transplantation, due to the relatively defined pathology affecting primarily midbrain dopamine neurons. At the onset of clinical symptoms the majority of midbrain dopamine neurons have already died, providing further rationale for a cell replacement approach. The first ES cell-based study that showed functional improvement in 6OHDA lesioned rats, an animal model of Parkinson's disease, was based on the transplantation of low numbers of largely undifferentiated mouse ES cells isolated after short-term differentiation in EB cultures. Spontaneous differentiation into large numbers of neurons with midbrain dopamine characteristics was observed. However, the clinical relevance of this approach is limited, due to the high rate of teratoma formation caused by the presence of undifferentiated cells in these grafts.

Remarkable functional improvement was obtained after transplantation of dopamine neurons derived from Nurr1 overexpressing mouse ES cells. This study reported behavioral restoration in 6OHDA lesioned rats, and demonstrated *in vivo* function via electrophysiological recordings from grafted dopamine neurons in slices obtained from the grafted animals. Another study showed behavioral recovery with dopamine neurons derived from naïve mouse ES cells. Both regular mouse ES cells and ES cells derived via nuclear transfer (ntES cells) from tail tip fibroblasts were used in this study. Successful grafting of ntES-derived dopamine neurons provided a first example of therapeutic cloning in neural disease. More recently it has been shown that this approach can be used successfully to treat individual mice with their matched ntES cell-derived dopamine neurons. This study provided definitive proof-of-concept of therapeutic cloning in an individualized manner, and showed the relevance of genetically matching DA neuron progeny for achieving optimal outcomes. DA neurons derived from mouse iPS cells have also been used successfully to treat a rat PD model, providing evidence for yet another source of pluripotent cells relevant for DA neuron replacement strategies. However, despite the successes with DA neurons derived from mouse pluripotent cells, the results with human cells have been moderate at best, suffering from poor survival and maintenance of DA neuron phenotype *in vivo*.

Huntington's Disease

Similar to PD, the pathology in Huntington's disease (HD) affects a selective neuronal cell population, the GABAergic medium spiny neurons in the striatum. However, unlike in PD, grafted cells are required to reconnect not to local targets within the striatum, but to project from the striatum to the targets in the globus pallidus and the substantia nigra pars reticulata. Recent studies have demonstrated the derivation of medium spiny GABA neurons from human ES cells *in vitro*. Upon transplantation these cells are capable of generating areas of DARP32+ striatal patches, suggesting survival and proper fate specification of ES cell-derived medium spiny neurons. However, translation of this approach is plagued by the presence of proliferating neural precursor cells within the graft that cause an overgrowth phenotype. Another interesting avenue for stem cells in HD is the derivation of hES cells from embryos with HD mutations identified during pre-implantation diagnostics or the establishment of disease-specific human iPS cells. Such HD model lines could provide invaluable

insights into the selective vulnerability of the striatal GABAergic cell population.

Spinal Cord Injury and Motoneuron Disorders

Traumatic or degenerative injuries to the spinal cord are often devastating and irreversible. Cell replacement using stem cells has been touted as a prime application of stem cell research. However, the complexity of cell therapy in spinal cord injury is enormous. Motor neurons are one of the main cell types affected in spinal cord injury, and in various degenerative diseases such as ALS.

Prior to the availability of directed differentiation protocols, there have been reports on functional improvement in animal models of spinal cord injury after grafting ES progeny. In one such study dissociated 4−/4+ mouse EBs derived from D3 or Rosa26 ES cells were transplanted into the spinal cord, nine days after a crush injury. The grafted cells differentiated *in vivo* into oligodendrocytes, neurons, and astrocytes, and induced significant functional improvement in BBB scores as compared to sham injected animals. However, the mechanism by which functional improvement was obtained remains controversial. More recent studies have suggested the ES cell derived motoneurons can project from the spinal cord towards peripheral targets, guided in a step-wise manner by appropriate attractive target factors. However, it remains unclear whether the modest extent of reinnervation achieved by ES-derived motoneurons can account for any functional changes in outcome. In spinal cord injury, the main therapeutic avenue is the transplantation of ES cell-derived oligodendrocytes promoting the remyelination of denuded axons. This strategy is under development as one of the first potential clinical applications of human ES cells, prompted by the ability to derive large number of cells expressing oligodendrocyte markers.

Stroke

Very little information is available on the behavior of ES cell progeny in animal models of stroke. It has been shown that grafted ES cells can survive in a rat stroke model induced by transient ischemia via occlusion of the middle cerebral artery. The goal of some of these early studies was the non-invasive imaging of the grafted cells using high resolution MRI after transfection with superparamagnetic iron oxide particles. More recently, human ES cell-derived neural precursors have been tested in a rat ischemic stroke model. Engraftment of the cells was associated with functional improvement in the animals. However, cell transplantation efforts for the treatment of stroke are complicated by the variability of the affected cell populations, depending on stroke location, and the enormous challenges in trying to recreate complex neuronal circuits *de novo* within the adult brain.

Demyelination

The capacity of mouse ES-derived progeny to remyelinate *in vivo* has been demonstrated after transplantation of highly-purified ES-derived glial progenitors into the spinal cord of *md* rats, an animal model of Pelizaeus–Merzbacher Syndrome. This study showed impressive *in vivo* differentiation results, and yielded large grafts comprised of myelinating oligodendrocytes. However, the grafted cells were not able to extend the short lifespan of these animals, precluding detailed functional analyses. A second study demonstrated remyelination after grafting purified oligosphere cultures derived from 4−/4+ EBs into the spinal cord of shiverer mice, or into the chemically demyelinated spinal cord.

While human fetal cells have demonstrated a remarkable potential for extensive remyelination in various animal paradigms, comparable data with human ES cell-derived oligodendrocytes remain less convincing to date.

Other Diseases

Several disease models that have been approached with fetal neural progenitors are currently being tested using ES cell-based approaches. These include epilepsy, and enzymatic deficiencies such as lyosomal storage diseases. Other CNS disorders, such as Alzheimer's disease, have been touted as future applications for ES cell therapy. However, at the current stage of research, the challenges for cell therapy in Alzheimer's disease seem to be overwhelming, and it appears more likely that the role for ES cells might be in providing cellular models of disease rather than for cell replacement.

CONCLUSION

The development of directed differentiation protocols provides the basis for all ES cell-based approaches in neural repair. Over the last year, enormous progress has been made in both mouse and human ES cells, and a broad range of neural cell types can now be routinely derived. However, it will be critical to perform more systematic studies to address lineage specification in ES cell progeny, and to define strategies for the prospective isolation of highly-purified neural cell populations. Access to purified cell populations will be critical for the development of ES cell-based transplantation approaches and ultimate clinical translation within CNS disease. Beyond the obvious potential in regenerative medicine, ES cells will also become a critical tool for gene discovery and for the study of lineage

specification during mouse and human neural development. With the advent of human iPS cell technology, and the development of HTS assays, one of the most critical applications for the future will be the use of pluripotent cells in disease modeling and drug discovery.

KEY WORDS

Embryoid body Clusters derived from aggregated embryonic stem cells. Embryoid body formation initiates a process that shares similarities with gastrulation, leading to differentiation of ES cells into cells of the three germ layers (ectoderm, endoderm, mesoderm).

Neural patterning Developmental mechanism to specify regional identity of neural precursor cells (e.g., forebrain versus midbrain versus spinal cord identity, or dorsal versus ventral identity). Neural patterning is often controlled by soluble proteins that act in a concentration-dependent manner to specify naïve progenitor cells.

Neural rosettes Term used to describe the tube-like columnar epithelial structures appearing during the early stages of neural differentiation from ES cells. These structures are particularly prominent during neural differentiation of primate ES cells. Rosettes correspond developmentally to the neural plate and early neural tube stage.

Neurospheres Non-adherent clusters of neural progenitor cells grown *in vitro*. Culture system for the propagation of neural stem cells.

Prospective isolation A strategy to isolate defined cell types based on a set of predetermined cell-specific markers using fluorescence-activated cell sorting (FACS), or other techniques that allow physical separation of cells.

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Chapter 20

Neuronal Progenitors in the Adult Brain: From Development to Regulation

Christian Mirescu and Elizabeth Gould

INTRODUCTION

The adult mammalian brain has vastly reduced regenerative potential compared to the developing brain. Nevertheless, cell proliferation occurs in the adult brain, as does neurogenesis, and cells that give rise to neurons and glia *in vivo* and *in vitro* have been identified in the adult central nervous system (CNS). As an understanding of the factors that regulate stem cell proliferation and neurogenesis in the adult brain deepens, the development of methods directed at repairing the damaged brain through transplantation of cultured cells, or through induction of endogenous neurogenesis in selected neuronal populations, may lead to novel therapeutic strategies. This chapter will review the evidence that neural stem cells exist in the adult brain, and discuss the factors that determine whether these cells divide and, if so, what is the fate of their progeny.

HISTORY OF STEM CELLS IN THE ADULT CENTRAL NERVOUS SYSTEM

Although studies dating back to the 1960s have documented neurogenesis in the adult mammalian brain, the isolation of cells with stem cell properties, i.e., multipotent and self-renewing, from adult neural tissue did not occur until much later. In the early 1990s, reports demonstrated that cultured cells from the adult rodent striatum produced both neurons and glia, effectively documenting multipotentiality. These initial findings by Reynolds and Weiss (1992) from the University of Calgary were followed by more specific localization of stem cells within this brain region, this time identifying proliferating cells as residents of the subventricular zone (SVZ), which may have been included in some of the early cultures since the SVZ borders the striatum. Since that time, numerous reports have identified cells from adult animals with the potential to produce neurons and glia when grown in culture or transplanted into other brain regions. In addition to the SVZ, such cells have been isolated from a variety of locations in the CNS, including the spinal cord, the hippocampus, and the neocortex, as well as the striatum. The self-renewing capability of these cells was reported in 1996 by McKay and colleagues from the National Institutes of Health (NIH), as well as Arturo Alvarez-Buylla and co-workers, demonstrating conclusively that these cells had the characteristics of stem cells when grown *in vitro*. Studies since that time have sought to identify stem cells *in vivo* with some success.

GLIAL CHARACTERISTICS OF NEURAL STEM CELLS

Beginning with studies of neurogenesis in adult birds, and then extending to those in adult and developing mammals, several *in vivo* reports have suggested that stem cells have the characteristics of glia. These observations further expand the burgeoning list of functions attributed to glia, and may lend insight into the production of neurons in the adult brain.

Radial Glia

In adult birds, the germinal zone of the lateral ventricles resembles that of the embryonic ventricular zone (VZ) of mammals. Adult-generated neurons appear to use radial glia processes as migratory guides to their final destination in the rostral forebrain. The spatial and temporal relationships of radial glia to new neurons were observations that led to the first suggestion that radial glia may themselves serve as stem cells. That is, radial glia may give rise not only to new glia, but also to neurons.

More direct evidence that glia serve as neural stem cells in the avian VZ comes from retroviral lineage studies, which have shown that radial glia exhibit multipotent differentiation. In the mammalian embryo, the VZ generates postmitotic cells that also take a rostral migratory route in

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forebrain development. Recent studies have shown that neuronal progenitors are radial glia in the embryonic mammalian forebrain. During subsequent neonatal development the radial glia of the VZ disappear, and are replaced by multiciliated ependymal cells that come to line the walls of the maturing lateral ventricles. Coinciding with this time, mitotically active cells begin to appear in the subventricular zone (SVZ). These dividing cells show ultrastructural characteristics of radial glia, and furthermore express the radial glial marker RC-2. That the postnatal SVZ progenitors share properties similar to the radial glia of the embryonic VZ suggests that the VZ may serve to seed the mature SVZ with a self-replenishing population of adult neural stem cells. In adulthood, the fate of SVZ cells resembles the movement of cells born during development, as postmitotic cells appear to migrate toward anterior regions of the brain along the rostral migratory pathway. Similarities in the site of origin, migratory route, and ultimate position of adult-generated cells in avian species suggest that processes active in embryonic development are conserved in germinal regions of the adult brain, and that radial glia may also be antecedents to adult neural stem cells in mammals.

Nevertheless, parallels drawn across species, and observations of structural similarities to embryonic VZ cells, are only indirect evidence that radial glia actually serve as primary neuronal precursors. More direct evidence supports this view: radial glia (isolated by fluorescent-activated cell sorting) have been observed to give rise to both neurons and astroglia. Furthermore, radial glia retrovirally-tagged with green fluorescent protein (GFP) in the developing rodent brain have been found to have mitotic activity, and to subsequently generate neurons. Such reports demonstrate more directly that radial glia progenitors are not an exclusive characteristic of the avian brain. In mammals as well, these cells also appear to be neural stem cells.

Despite the evidence that radial glia serve as progenitors in the adult avian and reptilian brain, as well as in the developing mammalian brain, it is less likely that new neurons arise from radial glia in the adult mammalian brain. This is because radial glia are absent in the mature mammalian brain, as they are thought to differentiate into astroglia following development. Thus, in adult mammalian germinal regions, a distinct type of progenitor must exist, perhaps descended from embryonic radial glia, which retains the self-renewing and multi-lineage potentials of neural stem cells *in vivo*.

Astroglia

Numerous recent studies suggest that progenitor cells in the adult mammalian brain share characteristics with astroglia. Temporary destruction of neuroblasts and immature precursor cells in the adult brain by treatment with an antimitotic agent spares some astroglia in the SVZ. Within hours after halting cell division, astroglia begin to divide, and then several days later, migrating neuroblasts destined for the olfactory bulb, and possibly other regions, emerge. Finally, by 10 days later, the orientation and organization of chains of migrating neuroblasts resemble that of normal adult mice. Thus, astroglia appear to function as neural progenitors *in vitro* and *in vivo*, and appear critical for recovery of germinal activity within the SVZ.

In addition to the SVZ as an identified region of adult germinal activity, the subgranular zone (SGZ) of the hippocampal dentate gyrus appears to contain cells with stem cell-like characteristics in adulthood. The SGZ lies between the granule cell layer (GCL) and the hilus of the dentate gyrus. Astroglia within the SGZ share ultrastructural features with those of SVZ astroglia. When dividing cells are labeled with [^{3}H]-thymidine, or with the thymidine analog bromodeoxyuridine (BrdU), in adulthood, a large proportion of adult-generated cells in the dentate gyrus exhibit an astrocytic phenotype soon after. The majority of BrdU-labeled GFAP-positive astroglia disappear rapidly, coinciding with an increase in the number of BrdU-labeled GFAP-negative cells, suggesting that SGZ astroglia may undergo a transition from a glial to a non-glial phenotype. In order to examine whether this transition actually gives rise to the production of new neurons in adulthood, antimitotic treatment was also used. Similar to the observed effects of such treatment in the SVZ, some astroglia survive and begin to divide soon after treatment. The administration of BrdU or [^{3}H]-thymidine two days after treatment, during SGZ astrocyte proliferation, yields labeled granule neurons, astroglia, and oligodendrocytes, both one and five months later. Thus, it appears that SGZ astroglia share neural stem cell properties similar to SVZ astroglia, namely, the capacity for self-renewal and multilineage differentiation.

ADULT NEUROGENESIS *IN VIVO*

Numerous studies have conclusively demonstrated the production of new neurons in the brains of adult mammals. The case for SGZ and SVZ as germinal regions for new neurons in the adult brain is very strong. Collectively, this evidence supports the view that new neurons (i.e., cells with morphological, ultrastructural, biochemical, and electrophysiological characteristics of neurons) are added to the dentate gyrus and olfactory bulb of adult mammals. Evidence for adult neurogenesis in other brain regions, including the neocortex, striatum, olfactory tubercle, amygdala, and substantia nigra, exists, but these findings await further investigation. In the light of *in vitro* studies demonstrating cells with stem cell-like characteristics in the parenchyma of regions other than the dentate gyrus and SVZ, adult neurogenesis in other brain structures would not be unexpected.

During development, neurogenesis is regulated by the complex interplay of peptide signaling molecules and neurotransmitters. Since neurogenesis persists in some regions after development is complete, it is possible that regulatory mechanisms of this process are also preserved. The following sections focus on the influence of various signaling molecules, such as growth factors, neurotransmitters, and hormones, on the production of new neurons in the adult brain.

Neurotrophins

Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are members of a structurally-related family of growth factors that function to prevent the death of embryonic neurons during development. Across the lifespan, neurotrophin expression varies dramatically. Embryonic expression of NT-3 appears highest in regions of the CNS where proliferation, migration, and differentiation are ongoing. Levels then decrease with maturation. By contrast, BDNF expression is lowest in developing regions, and increases with maturation. Both BDNF and NT-3 expression, and their preferred receptors TrkB and TrkC, have been demonstrated in cultures of cortical progenitor cells and *in vivo* at the onset of cortical neurogenesis, suggesting a possible direct action of these systems on neural progenitors. When regional BDNF expression is specifically enhanced by an adenoviral vector, a substantial increase of adult-generated neurons occurs in the mouse olfactory bulb, a known neurogenic region. In addition, BDNF overexpression results in adult neuron production in the caudate putamen, normally a nonneurogenic site in adulthood. By contrast, in hippocampal-derived stem cell clones, neurotrophins alone are only minimally effective in stimulating neurogenesis. Yet, in the presence of retinoic acid, both BDNF and NT-3 promote the acquisition of a neuronal fate by progenitors. These results suggest that neurotrophins may have regionally distinct and synergistic effects on adult neural stem cells.

Insulin-like Growth Factor

Neurogenic effects of insulin-like growth factor (IGF) have been identified in cultures of embryonic neural stem cells, as well as *in vivo*, using transgenic models overexpressing or lacking specific IGF encoding genes. In proliferative cultures of embryonic precursors, insulin and IGFs promote the proliferation of neural stem cells, and the acquisition of a neuronal phenotype. In mice and humans, disruption of the IGF-I gene is associated with profound retardation of brain growth. In contrast, overexpression of IGF-I results in larger brains marked by greater numbers of neurons. Similar effects on proliferation and neuronal lineage appear conserved in adulthood, as peripheral IGF-I infusion (for six days) increases the number of proliferating cells and the fraction of new cells exhibiting neuronal characteristics in the adult hippocampus of rodents.

Fibroblast Growth Factor

Extensive evidence suggests that basic fibroblast growth factor (bFGF) elicits neurogenic effects in culture, as well as in widespread regions of the prenatal brain, such as the cerebral cortex, the neonatal cerebellum, and the SVZ. When administered during adulthood, bFGF also exerts a mitogenic effect in both the SVZ and olfactory system. However, no effect of bFGF has been found in the adult hippocampus, suggesting that the germinal effect of bFGF might be ubiquitous during development, but regionally specific in adulthood. When bFGF is delivered to neonatal rats on P1, increases in hippocampal DNA content and enhanced production of BrdU-positive cells are observed beyond the early postnatal period. Thus, bFGF appears to influence olfactory and SVZ neurogenesis throughout life, but in the dentate gyrus only during development.

Glutamate

The bulk of excitatory inputs throughout the mammalian brain utilize glutamate as a neurotransmitter. The majority of inputs into the dentate gyrus GCL are glutamatergic afferents originating from the entorhinal cortex. Along with regions of the hippocampus other than the dentate gyrus, granule neurons of the dentate gyrus express *N*-methyl-D-aspartate receptors (NMDAr). In development, NMDA receptor blockade results in profound changes in the dentate gyrus and SVZ. Specifically, NMDA receptor antagonists increase cell proliferation in both germinal regions of rat pups. Similarly, in adulthood, both lesions of the entorhinal cortex and injections of either a competitive or noncompetitive NMDAr antagonist have been demonstrated to potentiate the birth and overall density of granule neurons. In contrast, injections of NMDA rapidly reduce the number of dividing cells in this region. However, a direct influence of glutamatergic neurotransmission on proliferating cells in the adult dentate gyrus is not likely, because granule cell progenitors do not express the NR1 NMDAr subunit, which is obligate for receptor function.

Serotonin

Recent evidence suggests that serotonin (5-HT) exerts an influence on adult neurogenesis. Inhibition of 5-HT synthesis via parachlorophenylalanine (PCPA) administration and selective lesion of serotonergic neurons reduce numbers of adult-generated cells in the dentate gyrus and the SVZ. Similarly, the selective blockade of 5-HT1a receptors potently decreases cell proliferation in the dentate gyrus,

suggesting that the serotonergic influence on adult hippocampal cell production is mediated (at least in part) by this high-affinity 5-HT receptor. Furthermore, treatment with a variety of antidepressants, which work (at least in part) through 5-HT, increases the production of new granule neurons in the dentate gyrus.

Adrenal Steroids

Recent studies have also identified numerous hormones that have regulatory effects on adult neurogenesis, with adrenal steroids as the most widely-researched neuroendocrine regulator of cell proliferation. In the dentate gyrus, reductions in circulating adrenal steroids produced by adrenalectomy are associated with increased numbers of proliferating cells within the adult dentate gyrus. In contrast, increases in exogenous corticosterone (CORT) reduce adult cell proliferation in the dentate gyrus of both neonatal and adult rats. In agreement with such results, various naturalistic stressors exert suppressive effects on cell proliferation in the prenatal, perinatal, and mature dentate gyrus. Such experiences reduce both proliferative activity and immature neuron numbers via an adrenal steroid-dependent mechanism that appears to operate via NMDAr activation. Recent evidence also suggests that chronic stress and adverse early life experiences exert more lasting effects on adult neurogenesis. In addition to stress, aging has also been associated with diminished adult cell production in adult rodents. Adrenalectomy in aged rats appears capable of restoring proliferative activity to levels similar to those of young rats, suggesting that adrenal hormones also mediate age-associated effects on the dividing dentate gyrus. Such effects, however, do not seem to underlie age-related cognitive decline in hippocampal-dependent tasks.

Ovarian Steroids

Ovarian hormones have also been found to influence the rate of cell proliferation in the dentate gyrus. In adult female rats, natural periods of high estradiol (proestrus) are associated with rapid increases in cell production. In contrast, at longer survival time points in both wild-reared and laboratory-reared meadow voles, cell proliferation and survival have been reported to be inversely related to the levels of circulating estradiol. Thus, it appears that estradiol exerts a transient stimulatory effect and a subsequent suppressive influence on dentate gyrus division. The estradiol-induced suppression appears to be mediated by adrenal steriods, as CORT levels increase in response to elevated estradiol.

SUMMARY

Studies carried out over the past decade have identified cells with stem cell-like properties in the adult mammalian brain. The bulk of evidence suggests that these cells exhibit characteristics of glial cells, a finding that may elucidate basic mechanisms of neurogenesis and make isolating stem cells a more tractable problem. The production of new neurons in the adult brain has been shown to be regulated by growth factors, neurotransmitters, and hormones. A more detailed understanding of the potential interactions among these modulators of adult neurogenesis may enable the controlled manipulation of neuron production in the damaged brain.

Numerous neurological conditions are associated with the loss of neural cells, perhaps most notably Alzheimer's disease and Parkinson's disease. The possibility that trauma and neurodegenerative disorders can be treated, either by transplanting neural stem cells, or by stimulating endogenous neurogenesis in existing populations of adult neural stem cells, has theoretically powerful clinical potential. The key advantage of transplanting cell lines is that they can be genetically engineered, allowing for customization depending upon the specific disease. On the other hand, strategies designed to enhance endogenous neurogenesis (perhaps with regional specificity) would have the potential for clinical efficacy without requiring invasive techniques. Although it is presently unclear which manipulations will lead to specific types of clinical improvements, what is certain is that the presence of neural stem cells in the adult CNS has opened up a new frontier of scientific exploration.

KEY WORDS

Adult neurogenesis The addition of new neurons to the adult brain, a process consisting of the proliferation, differentiation, and survival of neural stem cells.

Dentate gyrus Subregion of the hippocampus in the medial temporal lobe, which contains the subgranular zone, the neurogenic site of the dentate gyrus.

Neural stem cell Cell within the CNS which retains the capability of: (1) self-renewal; and (2) multilineage potential (i.e., ability to produce a variety of cell types; in the brain, predominantly neurons and glia).

Subventricular zone Region of the forebrain bordering the cerebral ventricles and identified as a neurogenic site.

Trophic factor Growth substance produced by target cells, which promotes the proliferation of progenitor cells and/or the survival of their neuronal progeny by preventing programmed cell death (i.e., apoptosis).

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Chapter 21

Sensory Epithelium of the Eye and Ear: Update of Retinal Stem Cell Research 2003 to the Present

Connie Cepko and Donna Fekete

INTRODUCTION

There have been advances in our understanding of Mueller glia (MG) as a source of new retinal neurons. MG are a very promising source of new neurons, as they are distributed throughout the retina and retain the gene expression profile of the retinal progenitor cells (RPCs) that produce all retinal neurons throughout development. Recent studies in fish and mammals have explored the response of MG to the loss of retinal neurons, particularly photoreceptor (PR) cells, and have demonstrated their ability to replace those cells. In addition, manipulation of ES cells to the point where PRs can be made *in vitro* has been reported. Recent reviews regarding the engraftment of various types of ocular cells for retinal repair have covered the potential and challenges faced by engraftment strategies (e.g., Limb, 2006; MacLaren, 2007). This section will cover the progress made towards the genesis of retinal neurons from embryonic or adult stem cell sources.

MUELLER GLIA AS A SOURCE OF NEW RETINAL NEURONS

As discussed earlier in this chapter, MG are among the last generated retinal cell types. Their morphology is similar to that of RPCs and of radial glia that function as progenitor and/or stem cells found elsewhere in the central nervous system. Molecularly, MG have been characterized using two comprehensive gene expression profiling methods: SAGE; and microarrays. Individual MG cells from mice were subjected to mild lysis, followed by reverse transcription and PCR. The resulting cDNA preparations were applied to comprehensive Affymetrix chips. The signals from five probes made from five single MG cells were then compared to similarly-prepared single cell probes from RPCs and a variety of retinal neurons (Roesch, 2008). As shown earlier by SAGE (Blackshaw, 2004), MG are very similar to RPCs. They retain the overall RPC profile, expressing the classic RPC genes, such as Sox2, Notch pathway genes, and cell cycle genes. In addition, at least some of them express Pax6 and Chx10, two other classic RPC genes. Expression of Pax6 at a low level in zebrafish retina has also been recently reported (Bernardos, 2007). Interestingly, Chx10, as well as several MG genes, are not expressed in all MG, suggesting that there is heterogeneity among MG, which might extend to variation in their abilities to generate retinal neurons (Roesch, 2008). All of these data point to MG as a logical source of retinal neurons. Curiously, however, as summarized above, early reports on retinal neurospheres did not report the production of neurospheres from the central retina, which includes MG, as a good source of neurospheres (Tropepe, 2000). Non-activated MG taken straight from an uninjured retina thus may not have self-renewal properties, and/or may not have the response that stimulates cell division under the neurosphere culture conditions. However, manipulations where the MG are first stimulated to divide in a particular culture condition, and then cultured in neurosphere conditions, allows them to form neurospheres (Das, 2006). Transplantation of these cells *in vivo* has demonstrated their ability to produce some neurons with retinal antigens, with expression appropriate to the location of the engrafted cells. Interestingly, when injured retinas are analyzed and sorted by fluorescence-activated cell sorting (FACS) prior to culture, cells with MG markers could be identified by the Hoescht dye efflux assay (i.e., they appear as a side population (SP) in the FACS). This assay has been used to identify such SP populations which have been shown to be stem cells in multiple tissues, and suggests that the MG isolated following injury have been induced to a stem cell state. Very few MG from non-injured retinas were recovered

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in the SP following FACS. Stimulation or inhibition of either the Notch or Wnt pathways showed that SP cells were increased by Notch and Wnt pathway activation, and decreased by inhibition of these pathways. Neurosphere generation was similarly affected. Again, the response to these factors suggests that MG have features in common with stem cells from other tissues.

More important than the *in vitro* behavior of MG is their ability to divide and produce neurons *in vivo*. This ability was demonstrated to a limited extent in the post-hatch chick. Retinal regeneration in fish (Hitchcock, 2004) has also been investigated regarding the role of MG in regeneration. Yurco and Cameron showed that cells with MG properties in the vicinity of a local mechanical ablation re-entered the cell cycle and produced neurons that populated the ablated region (Yurco, 2005). The newly-generated neurons appeared to use local MG for migration to form the appropriate layers. Fausett and Goldman generated transgenic fish with a GFP transgene driven by an alpha 1 tubulin promoter fragment whose retinal expression was specific to MG in injured retinas (Fausett, 2006). They found that GFP+ cells divided and were the source of neurons in all laminae of the regenerated retina. Similarly, using a GFAP-GFP transgenic fish, which labels MG only in the adult fish, Berbardos and Raymond (Bernardos and Raymond, 2007) showed that MG responded to the bright light ablation of PR by dividing and producing both rod and cone PR cells. The response was only in the ablated area and the regenerated area of the retina looked remarkably normal. Analysis of the molecular response to these injuries is ongoing (Fausett, 2008).

Mammalian MG also appear to participate in limited regeneration. The Takahashi laboratory treated rat retinas *in vivo* with the neurotoxin, N-methyl-D-aspartate (NMDA), at doses that kill ganglion cells and some cells of the INL (Ooto, 2004). They found cells with MG properties entered the cell cycle and produced neurons that migrated to various retinal laminae. The number of such neurons was quite small. However, they could stimulate the production of different types of neurons using either environmental cues or transcription factors delivered by retroviruses. The Chen laboratory found that treatment of retinas with a glutamate receptor agonist, alpha amino adipate, led to the proliferation of MG (Takeda, 2008). This agonist apparently acts directly only on MG, and it was administered at a dose that did not led to MG death, only to MG proliferation. The BrdU+ cells moved into the ONL, and differentiated into cells expressing rod PR markers, as well as exhibiting the nuclear morphology of rod PR cells. The amount of proliferation was quite extensive, yet was limited and did not led to an excessive number of cells. The laboratory of Takahashi further explored regeneration from MG in mammals (Osakada, 2007). They stimulated MG proliferation using Wnt3A, or a Wnt agonist. This led to the production of PR cells, particularly if they introduced the Crx gene, which, as shown in previous studies, led to the production of more cells with PR-specific genes.

The recent studies of regeneration by MG suggest that the stimulation of MG proliferation is a promising avenue for the development of therapies for the replacement of retinal neurons. In the many cases where a genetic defect leds to degeneration, a repair strategy can be undertaken. The MG can be genetically-treated so that their progeny were repaired, e.g., for defective PR genes. Thus, the PR cells so produced would presumably be functional and perhaps able to participate in retinal circuitry. Stimulation of neuronal replacement by endogenous MG would also bypass two problems faced by the engraftment of cells into the subretinal or vitreal space. Many engrafted cells do not enter the retina proper, but remain in the sub-retinal space or vitreous body (e.g., Singhal, 2008). This may be due in part to an environment that blocks their integration. The production of retinal neurons from within the retina by MG would presumably not led to this type of problem, although such neurons would still need to migrate to their proper position and, ideally, form proper synapses. Migration was demonstrated in the aforementioned studies, although synapse formation has not been demonstrated. An additional problem faced by engrafted cells is the problem posed by the immune system. It is not clear if there is long-term immune privilege in the retina, and thus it is not clear whether autologous tissue would be needed for engraftment. Again, regeneration from endogenous MG would bypass this problem.

EMBRYONIC STEM CELLS AS A SOURCE OF RETINAL NEURONS

ES cells have been explored as a source of replacement neurons for retinal degenerative diseases. The cell types most often in need of replacement are PR and ganglion cells, although in some cases RPE cells will need to be replaced. Cells expressing markers of RPE (Kawasaki, 2002; Hirano, 2003), lens (Ooto, 2003; Hirano, 2003), and retina (Ooto, 2003; Hirano, 2003; Osakada, 2008; Lamba, 2006) have been generated from ES cells *in vitro*. For example, Hirano *et al.* (2003) showed that murine ES cells cultured with the stromal line, PA6, developed into remarkably well-organized aggregates with eye-like layers, and expressed markers of lens, retina, and RPE. Culturing with PA6 and dexamethasone, cholera toxin, and bFGF increased the yield of such structures (Hirano, 2003). When injected into the early chick eye, murine ES-derived cells were observed to express markers of RPE (Aoki, 2006). When co-cultivated with retinas taken from mice treated with the neurotoxin, NMDA, these ES-derived eye-like structures integrated into the damaged retina, and some cells with ganglion cell markers were detected (Aoki, 2007). More importantly, some cells with ganglion cell features were observed when these cells were injected into NMDA-injected adult eyes (Aoki, 2008).

Tabata *et al.* showed that more integration, and the development of cells with ganglion and horizontal cell antigens, was achieved if ES cells from feeder-free cultures were first transformed by Rax, and then subjected to retinoic acid (RA) treatment, prior to co-culture with retinal explants (Tabata, 2004). Encouragingly, some of the engrafted cells had electrophysiological properties of ganglion cells. As Rax is an early gene expressed by RPCs (Furukawa, 1997; Mathers, 1997), and loss of Rax leds to the loss of eye formation (Mathers, 1997), this result is in keeping with the induction of retinal identity in the ES cells.

Ikeda *et al.* (2005) showed that initial treatment of murine ES cells with a Wnt antagonist (dkk1) and a nodal antagonist (leftyA), followed by treatment with a BMP antagonist (noggin) and FCS, led to approximately 6% of all cells expressing Rax and Pax6, markers of RPC. ES cells treated in this way were more responsive than untreated ES cells to the introduction of Crx, a PR transcription factor. Following infection with a lentivirus encoding Crx, Rax-transformed cells had rhodopsin expression in 21% of infected cells versus 0.8% of control infected cells. In addition, co-culture of treated ES cells with retinal cells in a mixed aggregate led to ES-derived cells that expressed rhodopsin, particularly when they were adjacent to host PR cells. They also co-cultured induced ES cells with embryonic retinal organ cultures (explants). Differentiation of some of the ES cells into rhodopsin-expressing cells was seen when the ES cells resided in the outer nuclear layer, as appropriate for PR cells which normally reside in the outer nuclear layer. In addition, some markers of MG and bipolar cells were seen by cells within the inner nuclear layer, and a few expressed ganglion cell markers when they resided in the ganglion cell layer. This strategy has been recently extended to human ES cells (Banin, 2006; Lamba, 2006; Osakada, 2008). Banin *et al.* (2006) found that human ES cells treated with noggin, followed by culture with EGF and bFGF, led to the formation of neurospheres with expression of RPC genes, along with some markers of retinal neurons, including several PR markers. Engraftment of these cells into rat retinas led to little integration of ES derived cells, although a small percentage of the integrated cells did express markers of retinal neurons. Treatment of human ES cells with an inducer, insulin-like growth factor 1 (IGF-1), along with antagonists of the BMP and Wnt pathways, followed by culture with bFGF, led to the induction of RPC genes in almost all ES cells (Lamba, 2006). Dissociation of such cells and further culture led to cells with some antigenic and physiological characteristics of inner retinal neurons, the ganglion and amacrine cells. A few cells expressed markers of immature PR cells. Co-culture of the ES-derived cells with retinas from mice where photoreceptors degenerate led to the induction of more cells with markers of PR cells. Overall, these studies showed that ES cells could be used as a source of PR cells, but they did not led to a large number of cells with PR markers or morphology.

Osakada *et al.* (2008) have been able to significantly increase the number of ES-derived cells that differentiate into rod and cone PR, without the need for a co-culture with retinal tissue. They used a step-wise treatment with factors known to induce PR cells, most effectively employing DAPT, a gamma secretase inhibitor known to inhibit Notch activation. As loss of Notch1 has been shown to led to the exit of RPC from the cell cycle and the genesis of more PR cells, Notch1 has been proposed to act in RPC at the earliest stage of PR genesis (Jadhav, 2006; Yaron, 2006). The DAPT-treated ES cells were found to become both rod and cone PR cells, expressing many markers of PR cells, including the late-stage opsin genes. The number of cells expressing such markers was increased when the DAPT-treated cells were further treated with several factors previously shown to enhance PR development, including sonic hedgehog, taurine, aFGF, bFGF, and RA. This work was extended from mouse ES cells to both monkey and human. In the case of human ES cells, up to 8.5% expressed rhodopsin, while 8.9% and 9.4% expressed the red/green and blue cone opsin genes, respectively. RPE cells also could be generated from the ES cells, using leftyA, or dkk1.

Stem cell engraftment as a means of replacing degenerating retinal cells will continue to be explored given the lack of effective therapies for the majority of retinal degenerations. The challenges listed above concerning the engraftment site, which will likely be quite compromised, and perhaps inhibitory to integration and differentiation, as well as the specter of immune system rejection, will need to be addressed. In addition, the function of engrafted cells, through the formation of appropriate synapses, or via trophic support of residual host cells, will need to be established. The fact that ES cells, or adult stem cells, can be induced to form cells with PR markers is encouraging. The observation that engrafted cells tend to express genes appropriate to their location, either due to the induction of such programs in RPC, or due to the homing of cells with particular identities to their appropriate layers, is also a hopeful sign. In addition, the recognition that injured or diseased retinas provide a host environment more conducive to induction leds to the notion that there are environmental cues that might be discovered that should led to a more effective production of the desired cell types.

KEY WORDS

Ciliary margin The peripheral region of the eye where retinal stem cells reside. In fish and amphibians, retinal cells are generated in vivo from these stem cells throughout life. It is also the area from which retinal stem cells have been isolated in mammals.

Otosphere Spherical colonies generated by dissociation and culturing of inner ear sensory cells from the newborn rat organ of Corti.

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Chapter 22

Epithelial Hair Follicle Stem Cells

Tudorita Tumbar and Elaine Fuchs

INTRODUCTION

Skin is a complex tissue made of a structured combination of cell types. It has an enormous regenerative capacity and contains several different populations of stem cells (SCs), including those that reside within specialized niches in the hair follicle, sebaceous gland, and epidermis. In addition, the hair follicle contains a different population of neural crest-derived melanocyte stem cells responsible for hair pigmentation. In this chapter we focus on epithelial skin stem cells, with special emphasis on the hair follicle stem cells (HFSCs) in postnatal mouse skin. Nonhuman studies allow for high flexibility and increased depth, and are less restricted by ethical issues. HFSCs reside in a structure within the outer root sheath (ORS) of the hair follicle called the "bulge." Cells in this compartment exist in a relatively undifferentiated state, where their normal role is to regenerate the hair follicle and regrow hair shaft with each hair cycle. However, during injury, they can contribute to repair of the sebaceous gland (SG) and the epidermis. In adult skin, bulge cells are relatively infrequently-dividing cells that preferentially retain nucleotide label over time. How these cells maintain their properties of self-renewal and differentiation remains largely unknown. Whether these cells are fundamentally different from their progeny, or whether it is simply their location, the so-called niche, that instructs them to behave as stem cells, remains an open question. Recent work has begun to elucidate signaling mechanisms that control the fate of these cells in mouse skin. The challenge to isolate infrequently-dividing cells and to identify markers of HFSCs has been overcome, enabling the tracing of the origins of bulge cells to embryonic skin, and opening new and exciting avenues for future insight into understanding how the niche develops and what maintains these cells in a dormant but potent state.

A BRIEF INTRODUCTION TO MOUSE SKIN ORGANIZATION

The complex process of constructing the protective cover of the body starts around day nine of mouse embryonic life (E9). Through a succession of signal exchanges between the ectoderm and mesoderm, a very structured tissue emerges designed to seal and protect the body of the animal against a diverse range of environmental assaults. The barrier function, or the sealing of the body from the external environment, is essential for the survival of the animal and is fully completed at E18, the day before the mouse is born. Hair follicle morphogenesis starts around E13, and takes place in waves until just after birth. The hair follicles are specified embryologically, and, consequently, the maximum number of hair follicles that an animal will have for the rest of its life is determined before birth.

Mature skin is composed of two main tissues: the epidermis and its appendages, largely composed of specialized epithelial cells (keratinocytes); and the dermis, largely composed of mesenchymal cells. Epidermis consists of an innermost basal layer (BL) of mitotically active keratinocytes that express diagnostic keratins K5 and K14. As these cells withdraw from the cell cycle and commit to a program of terminal differentiation, they remain transcriptionally active and move upward towards the skin surface. As they enter the spinous layers, they first protect themselves by switching from expression of K5 and K14 to K1 and K10, which form keratin filaments that bundle and provide a robust inner strength to the cells. As they complete this process, they next go about producing the barrier by synthesizing and depositing proteins, such as involucrin, loricrin, SPRR, and others, beneath the plasma membrane. In addition, as the cells enter the granular layer, they begin to produce and package lipids into lamellar granules, and they produce filaggrin, which bundles the keratin filaments

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even further to form cables. As the cells complete these tasks, an influx of calcium activates transglutaminases, which cross-link involucrin and its associates into a cornified envelope that serves as a scaffold for organization of the extruded lipids into external bilayers. Metabolically inert, these cells undergo an apoptotic-like loss of their nuclei and organelles, resulting in flattened, dead squames. These highly-keratinized cells provide a seal to the body surface from which they are eventually sloughed, replaced by differentiating cells that are continuously moving outward. Epidermis regenerates itself every few weeks, and has a remarkable ability to heal, expand, or retract in response to environmental cues.

Epidermal appendages, including the hair follicle and the sebaceous gland (SG) (Figure 22-1), are inserted deep into the dermis. The hair follicle is a very complex structure made of at least eight different cell types. The hair shaft is located in the middle of the follicle and grows upward, "breaking" the surface of the skin. Concentric layers of cells surrounding the shaft form the outer and inner root sheaths (ORS, IRS) (Figure 22-1). The basal layer of the epidermis is contiguous with the outer root sheath (ORS) of the epidermis, which the IRS degenerates in the upper portion of the follicle, liberating the hair shaft.

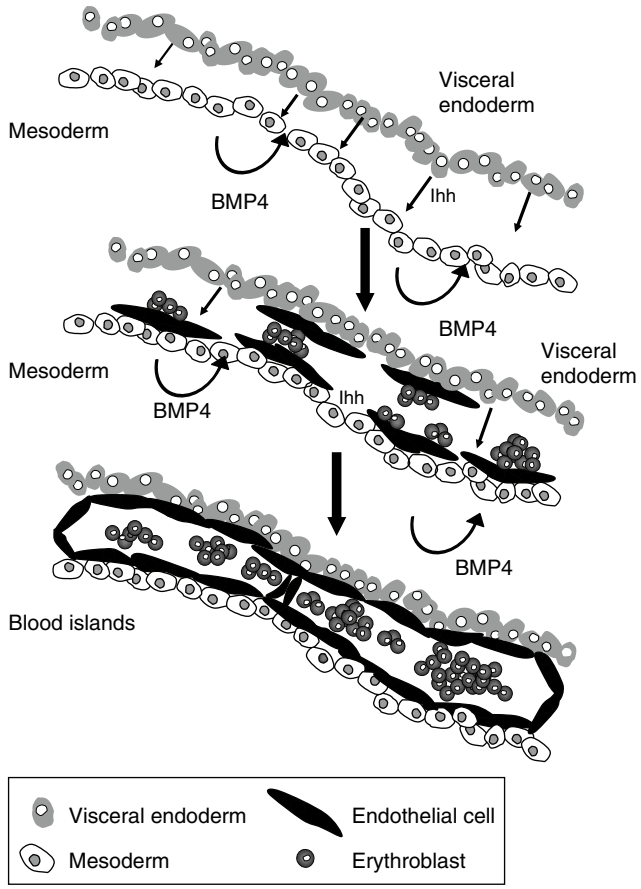


FIGURE 22-1 The hair follicle cycle in adult mouse skin.

The basal epidermal layer and the ORS share a number of common biochemical markers, including K5, K14, and $\alpha 6\beta 4$ integrins, and they represent a potent proliferative compartment of the skin. The highest proliferation, however, is seen in the matrix cells at the bulb of the follicle, which generate the IRS and the hair cells. Matrix cells surround a pocket of specialized mesenchymal cells, the dermal papilla (DP), with potential hair growth inductive properties. Matrix cells express a number of transcription factors (e.g., Lef1 and Msx-2) implicated in hair follicle differentiation. To some extent, IRS cells resemble epidermal granular cells, but their granules are uniquely composed of trichohyalin. In contrast, hair cells are more similar to stratum corneum, because they are metabolically inert as they complete differentiation; however, they express a unique set of hair-specific keratins. Finally, the SG is located in the upper portion of the hair follicle, just above the arrector pili muscle. The mitotically-active cells of the SG express K5 and K14. Sebaceous glands rely on their own stem cell population, which are marked by their specific expression of the transcriptional repressor Blimp1. Resident sebaceous gland stem cells differentiate into lipid-producing sebocytes that will subsequently self-destruct and release their oils (sebum) into the hair canal.

Hair follicles begin to form during late embryogenesis, and approach maturity by approximately day four of postnatal life, when hair shafts start to appear at the skin surface. In postnatal life, the ORS of maturing follicles widens a bit on one side, creating a bulge. Located below the SG, and near or at the juncture of the arrector pili muscle, this specialized region is thought to be the compartment where epithelial stem cells may reside. The bulge is marked by the embryonic expression of the transcription factors NFATc1, Lhx2, TCF3, and Sox9. In the adult, these markers are accompanied by CD34 and Keratin 15 promoter activity, which appear shortly before the first postnatal hair cycle. Recent functional studies reveal a functional role for Sox9-expressing bulge cells in completing skin morphogenesis in the embryo, and in fueling hair regeneration in the adult.

Stem cells in postnatal skin are required for self-renewal of the epithelial tissue. Like epidermis, cells of the postnatal hair follicles are in a state of flux, undergoing perpetual cycles of growth (anagen), regression (catagen), and rest (telogen). In relative synchrony, at least for the early hair cycles, mouse hairs continue to grow and complete anagen. Around 15 days of postnatal development, the first anagen, begun during embryogenesis, ceases. At this time, massive cell death is initiated in the bulb of the hair follicle, as the catagen phase begins. Follicle cells below the bulge area are destroyed, except for the DP, which moves upward by virtue of its attachment to a shrinking basement membrane that separates the epithelial and mesenchymal compartments. By around postnatal day 19 (P19), the DP comes to rest just beneath the

bulge. At this stage, hair follicles enter a brief telogen. The new anagen is reinitiated at around P21, and hair follicles again start performing this precise choreography of changes. Thereafter, anagen and catagen phases remain roughly similar in length, while the next telogen phase lasts over three weeks, and the next one afterwards is even longer. Signals from the surrounding environment, at least in part governed by BMP signaling, and yet unidentified signals from the DP are necessary to reactivate stem cells to restart the hair cycle. It remains a mystery as to why it takes increasingly longer to activate HFSCs with age.

Adult mouse skin contains epithelial stem cells located in at least three different compartments: epidermis, sebaceous glands, and hair follicle. This review focuses primarily on the hair follicle stem cells located in the bulge area of the hair follicle. Despite their location in the hair follicle ORS, cells within this compartment have been shown to give rise to all the different hair cell lineages, the SG, and the epidermis under injury conditions, although they remained confined to the hair follicle during normal tissue homeostasis.

THE BULGE AS A RESIDENCE OF HAIR FOLLICLE STEM CELLS

It has been postulated that stem cells should have evolved special mechanisms for protecting their DNA against accumulation of replication errors, which may otherwise result in a high rate of tissue cancer. These mechanisms may involve either slow or rare cell cycling and/or asymmetrically segregating the newly-synthesized DNA into the non-stem cell progeny (the immortal DNA strand hypothesis).

In a classical nucleotide pulse-chase labeling approach, a population of slow-cycling and/or asymmetrically dividing cells, likely to be the stem cells, is detected in the skin epithelium. When mouse pups are administered tritiated thymidine or BrdU from three to six days of postnatal life, the label is incorporated into the newly-replicated DNA, and is found abundantly distributed in the BL and ORS. When labeling is subsequently followed by four to eight weeks of chase, transient amplifying cells quickly divide and dilute the label, as they move up into the differentiated layers of hair follicle and epidermis, eventually being eliminated from the tissue. In contrast, the slow-cycling cells retain the label and are kept within the tissue. Through this approach, label-retaining cells (LRCs) have been localized, predominantly to the bulge area of the murine hair follicle, with fewer scattered cells in the basal layer of the epidermis or elsewhere in the epidermis or hair follicle. If the LRCs in the skin epithelium are stem cells, then the bulge represents a major stem cell compartment of postnatal skin epithelium.

More recently, a novel strategy was developed to fluorescently tag and isolate infrequently-cycling stem cells in tissues. The method was based on driving expression of a green fluorescent protein (GFP) fused to histone H2B in a cell type-specific and tetracycline-regulatable fashion. When expression is shut off, all dividing and differentiating cells dilute out the label, and/or are sloughed from the tissue, leaving only the infrequently-cycling cells brightly labeled. The system was successfully used in skin to monitor the label-retaining cells (LRCs) in the bulge, and to isolate and characterize these cells. Most recently, this system has been refined to count precisely the number of cell divisions during the first two synchronous hair cycles. Bulge cells, as defined by their cell surface expression of CD34 and α6-integrin, divide on average three times per hair cycle. A fraction of $\sim$30% of these cells divide only 0–4 times over two consecutive hair cycles, and these cells are the ones retaining the long-term BrdU label. There were no BrdU-bright bulge cells with low H2B-GFP levels, indicative of multiple divisions, contradicting the predictions of the immortal strands hypothesis. Moreover, when the BrdU label was quantified in cells with increasing number of divisions, it was found to constantly diluted by two-fold from one sub-population of dividing cells to the next generation of cells. Along with recent studies employing parallel methodology, these data argue against the immortal strand hypothesis in the hair follicle, and definitively establish the bulge as an infrequently-dividing cell population. Although blood, hair follicle, and epidermis do not appear to use immortal strand segregation as a means of regulating their stem cell populations, it should be noted that the hypothesis has received support from studies in brain, mammary gland, and muscle. It remains to be established whether different tissue stem cells protect their genomes by different mechanisms, or whether lack of quantitative methods, injury-induced artifacts, or other technical discrepancies are responsible for these differences.

With BrdU as a means of labeling the bulge cells, LRCs have been shown to contribute to the formation of both lower hair follicle and the upper region of the hair follicle ORS, called the “infundibulum.” This is based on the observation that LRCs can be found exclusively in the bulge area during the telogen phase of the hair cycle but, on initiation of a new anagen, cells containing less BrdU signal, presumably derived from division of bulge cells, were also found in the lower and upper ORS, matrix, and even medulla (in the hair shaft). The correlation with the bulge was indirect with a single-label experiment. Using a double-label technique that marked the upper ORS cells based on distinct division time, Lavker and colleagues were able to show a flux of the infundibular (upper follicle above the bulge) cells into the basal layer of the epidermis in neonates and in wounded adult skin.

By using the tetracycline-regulatable H2B-GFP pulse and chase system, LRCs were both efficiently and sufficiently brightly-labeled such that progeny could be tracked for about eight divisions. At the start of the new hair cycle (early anagen), transiently amplifying progeny of bulge LRCs could be found in the matrix in the follicle bulb.

Remarkably, even the terminally-differentiating hair and inner root sheath cells could be assigned on the basis of fluorescence as being cellular derivatives of the bulge. Moreover, in response to a wounding stimulus, these H2B-GFP LRCs appeared to exit the bulge and migrate toward the wound site. In transit to replenish the damaged epidermis, these cells also appeared to proliferate, deposit a fresh basement membrane, and change some biochemical properties.

The bulge area of the rat vibrissae has been shown to contain multipotent cells that have tissue morphogenesis ability. Micro-dissected rat vibrissae bulge tissue and cells transplanted onto the back of athymic mice yielded entire hair follicles, SG, and epidermis. Bulge cells also exhibited the highest colony-forming ability when cells were placed in tissue culture, and this was true regardless of hair follicle cycle stage. In other follicle regions, efficient colony-forming and morphogenetic cells were not found except for the bulb of the rat vibrissae, where such cells were detected only in early anagen.

In a recent study by Blanpain and colleagues (2004), bulge cells isolated from mouse pelage follicles harboring the actin-GFP transgene were plated at limiting dilution and grown in a culture dish. Single colonies were isolated, and the descendants from these individual bulge-cell colonies were mixed with newborn dermal cells and grafted onto the back of *Nude* mice. The newly forming skin contained epidermis, sebaceous glands, and entire hair follicles harboring the actin-GFP transgene, which demonstrated their derivation from an original bulge cell. These experiments not only suggest multipotency and the self-renewing properties of bulge cells, but also indicate their preservation during tissue culture expansion, an important requirement for future clinical applications. Further studies demonstrated the long-term self-renewing potential of single bulge cells, when their progeny were serially transplanted over a period of about one year. Most recently, the emergence of the infrequently-dividing nature of bulge cells was traced to embryonic skin, where Sox9-positive LRCs began to appear near birth. This neonatal niche within the ORS marks the development of the adult bulge niche, as some embryonically-labeled LRCs divided sufficiently infrequently to still be detected in the adult bulge.

Using an inducible Cre recombinase driven by a fragment of the *Keratin 15* promoter, expressed by the postnatal bulge, lineage-tracing experiments have demonstrated that *in vivo*, in the absence of injury or removing the cells from their natural environment, all the hair lineages derived from these cells. Moreover, when the K15+ cells were ablated using an inducible "killer" gene, the hair follicles could not be regenerated. The epidermis and sebaceous glands could survive in the absence of these cells, demonstrating their autonomy in the skin lineages. Corroborating results were obtaining using a Cre-inducible system driven by the *Shh* promoter, and for embryonic skin, using a *Sox9* promoter driven Cre.

There is a paradox between the infrequently dividing feature of bulge stem cells in skin, and the ability of cultured cells isolated from the bulge region to form large colonies over a two-week period. *A priori* it is possible that stem cells in culture divide more frequently, either through the trauma of the isolation process or through their exposure to culture medium. Although the formation of hair follicles from grafted cells indicated that each colony *in vitro* must have some expansion of stem cells, the bulk of each colony may be rapidly-dividing TA cells derived from these stem cells.

It is not yet clear precisely what relationship exists between LRCs identified in the bulge and the cells dissected from the bulge that give rise to cloned colonies or grafted follicles, although the majority of the cells used in such assay do display LRC characteristics. There is some indication, however, that LRCs are the cells that form colonies in culture. In a recent commentary, C. S. Potten cautioned about the use of *in vitro* approaches for stem cell studies. The study of stem cells outside of their tissue has been called by Potten "the biologic version of the Heisenberg principle." In other words, properties attributed to stem cells (i.e., colony forming or grafting) cannot be studied "without altering the tissue, and in so doing altering the stem cell state of the tissue." Conversely, however, it is equally plausible that most LRCs are innocent bystanders in the process and may never participate in the stem cell self-renewal or hair follicle morphogenesis. It is also worth noting that it has not been determined whether the infrequently-dividing feature of follicle stem cells is essential for their stemness. Thus, although intriguing as a potential stem cell marker, label retention must still be considered circumstantial and clearly dependent on the label-chase scheme used. Future studies will be necessary to evaluate this possibility, and to assess whether additional stem cells that are not label-retaining exist within the follicle.

An important study using retroviral transduction of keratinocytes has revisited the concept that the bulge contains a multipotent population of stem cells that is the normal source of tissue regeneration. In one study, Ghazizadeh and colleagues dermabraded mouse skin and transduced it with a retrovirus encoding the β-galactosidase reporter gene, followed by a 36-week chase with five cycles of depilation-induced hair follicle cycles. If the entire skin epithelium is generated from multipotent, long-lived stem cells within the bulge, then the distribution of β-Gal positive cells might be expected to be uniform across the different cell lineages in the hair follicle and epidermis. The result was puzzling because, even after the long chase and repeated stimulation of stem cells, only 30% of the hair follicles were uniformly blue. The rest of the follicles were positive in the ORS, IRS, or SG, but not in all three locations. Moreover, there were defined units of blue in the epidermis far away from the hair follicle. Similar results were obtained when the skin of mosaic mice obtained by

aggregation of two stem cell types of different genetic background was analyzed.

These results leave the door open to the notion that there could be multiple classes of stem cells that are long-lived, each with restricted potency that may not even reside in a single niche (the bulge). However, it seems equally plausible that the follicles with mosaic patterns of β-Gal expression may arise from perhaps more easily-infectible cells that have left the niche and are already committed to a particular lineage. The inability to infect non-dividing cells efficiently with retroviruses is well-established, and the extent to which infrequently-dividing cells were infected has not been adequately evaluated. Yet, the survival of the labeled cells during repeated rounds of hair growth stimulation, and their continuous contribution to differentiated tissue, attested to the stemness characteristics of this retroviral-labeled population. Another contributing factor may be that the mosaicism arises from a chromatin inactivation mechanism, referred to as position effect variegation, which often operates to spontaneously silence retroviral or transgene promoter activity. This mechanism may operate differently in stem cells and their committed progeny, leading to a nonrandom silencing pattern of gene expression of the reporter, in which the transduced retroviral DNA is silenced with higher efficiency in stem cells.

Despite these unresolved issues, the evidence is compelling that: (1) the bulge contains a pool of LRCs that participate in the regeneration of the hair follicle and of the wounded skin; (2) bulge cells can form large colonies in culture; (3) single-bulge cell progeny can regenerate entire hair follicles, SG, and epidermis; and (4) the Keratin 15 positive hair follicle cells regenerate the entire hair follicle in intact skin. The bulge cells have many of the characteristics expected of epithelial skin stem cells. Keeping the "principle of uncertainty" in mind, we now consider the different models of stem cell activation and stem cell function in the skin epithelium.

MODELS OF HAIR FOLLICLE STEM CELL ACTIVATION

Early data on hair follicle growth and stem cell function suggested that the bulb of the hair follicle, containing matrix cells, is the residence of stem cells. This hypothesis was hard to explain, because of the cycling nature of the bulb which undergoes extensive apoptosis in catagen, leaving only a small strand of epithelial cells connecting the DP and the bulge. In addition, the hair follicle can completely regenerate even after the bulb is surgically removed.

Evidence that the bulge contains a large pool of LRCs, together with the appealing location of the bulge as a "niche" at the base of the permanent portion of the hair follicle, suggested a novel model for hair follicle growth, the "bulge activation hypothesis" (Figure 22-2). In this model, stem cells are activated by yet-unidentified signals transmitted through direct contact with the DP. Such contact occurs at the end of each hair cycle, as the surrounding dermal sheath shrinks during the apoptotic phase and drags the DP upwards until it comes into contact with the bulge. Although this contact does not appear to be the sole source of stimulation, it seems to be a necessary stimulus to activate one or more bulge cells to divide, resulting in TA cells with decreasing levels of stemness.

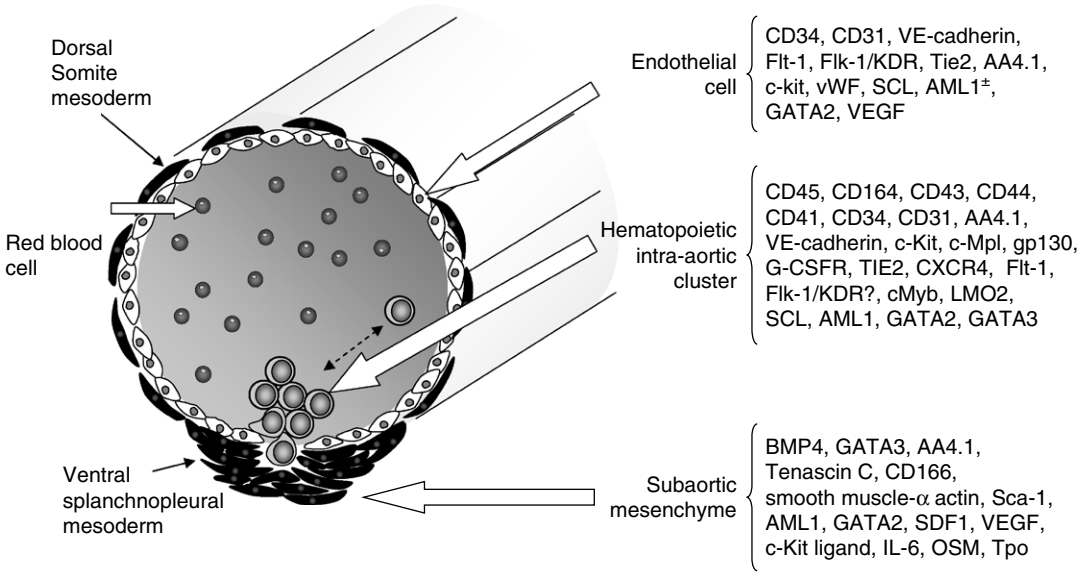


FIGURE 22-2 Models of stem cell activation in the hair follicle.

At least in neonate and wounded adult skin, stem cell activation may be accompanied by a flux of upper ORS cells to the epidermis. When cells exit the bulge and migrate upwards, they take on the fate of a TA epidermal or SG cell. When they migrate downwards during early anagen, they give rise to a population of TA ORS and matrix cells that in turn further specialize to form the IRS, cortex, and medulla. When matrix TA cells cease dividing, perhaps through exhaustion of their proliferative capacity, the bulb of the hair follicle undergoes apoptosis (catagen phase). Mutations in a number of genes, including those encoding the transcription factors hairless and RXRα, result in a failure of the DP to be dragged upwards at the end of the first postnatal hair cycle. The consequence is a block in bulge activation, and a loss of all subsequent hair cycles. The bulge activation hypothesis does not in itself explain why bulge cells and DP can sometimes sit adjacent to each other for extended periods of time without stem cell activation, but further experiments may bring more evidence in support of this model.

A second model for stem cell activation is the "cell migration" or the "traffic light hypothesis." This model is based on the observation that clonogenic and morphogenic cells in the rat vibrissae are found in the bulge at any hair follicle stage, but they are found at the base of the bulb in late catagen and early anagen. The telogen phase is short, if not absent, from vibrissae, and a new vibrissae cycle is initiated before the DP has moved upwards completely to come into contact with the bulge. In investigating this process, (2001) discovered that stem cells, or their more or less committed progeny, appeared to migrate along the ORS to come in contact with the DP, which then seemed to signal their pathway to differentiate. During catagen and early anagen, these bulge cell derivatives accumulated at the base of the hair follicle in a relatively undifferentiated state. Later in anagen, this "traffic stop" appeared to be removed, and cells at the base of the bulb seemed to progress to become matrix and then differentiate into IRS and hair shaft cells. While in transit along the basement membrane surrounding the ORS, these cells were not able to form colonies or engraft successfully, perhaps because of their low concentration. In this model, stem cells or their immediate progeny leave the niche, and with an estimated speed of ~100 μm/day, they migrate with minimal division along 2 mm of ORS to reach the bulb (Figure 22-2). Support for this model comes from short pulse-chase experiments on neonatal mouse backskin, and from analyses of skin development in Sox9-Cre mice crossed to the Rosa26-lacZ lineage tracing reporter mice.

A third model derives from an integrative reassessment of the literature, with a novel interpretation of the accumulated data. Known as "hair follicle predetermination," this model is based on multiple studies of hair follicle growth, and although speculative, it offers an alternative explanation for conflicting results. In this model, during mid- and late anagen, SCs are stimulated to leave the bulge niche and migrate away to form the ORS, but not the matrix or the rest of the hair. It is postulated that these SC progeny then accumulate at the base of the follicle, adjacent to the DP, where they are modified to form a "lateral disc" in the bulb (Figure 22-2). During telogen, the lateral disc maintains contact with the DP, and upon the next anagen, it proliferates (upwards) and forms the new matrix and the inner layers of the hair follicle. During this phase, newly-activated bulge cells give rise to the ORS and replenish the lateral disc. Therefore, the new hair follicle is formed from lateral disc cells that are predetermined for this role during the previous hair cycle. Based on the recent H2B-GFP LRC study in adult mice, it would seem that a modification of the predetermination model is now warranted to explain how, possibly as a secondary step following the initial "lateral disk" activation, the H2B-GFP LRCs are stimulated to exit the bulge area, proliferate, and participate in the formation of the matrix, all within a single anagen. Based upon additional pulse-chase studies, Cotsarelis and colleagues contend that the secondary hair germ emerges as a result of migration of stem cells out of their niche.

MOLECULAR FINGERPRINT OF THE BULGE: PUTATIVE STEM CELL MARKERS

One of the major problems in the advancement of the stem cell field has been the lack of specific biochemical markers characteristic for stem cells, despite extensive searches. This has raised the possibility that stem cells may have few, if any, unique features, but instead it is their niche that instructs stem cells to behave differently from their offspring. Recently, bulge cells have been isolated based on their CD34 or Keratin 15, as well as based on their infrequent division patterns as given by the H2B-GFP retention, and their transcriptional profile has been compared with progeny in the basal layer of the epidermis and ORS of the hair follicle. Overlapping results now provide tens of genes as candidate factors for bulge stem cell regulation.

Although a detailed analysis of all these newly identified factors will take time to complete, immunofluorescence microscopy has already indicated that a number of the proteins encoded by these bulge-up-regulated mRNAs are expressed within the bulge, but are not necessarily specific for the brightest LRCs within the bulge. Based on the comparison made, these mRNAs cannot be considered as exclusive markers for the bulge, and indeed some are expressed in other cell types within the skin. In addition, recent isolation and characterization of bulge cells attached to the basement membrane, versus those residing internally, have revealed heterogeneity in the molecular profiles of bulge cells. Whether these differences relate to a natural progression of bulge cells from attached, to detached, to exit/commitment is a fascinating possibility as yet unexplored.

Traditionally, some of the best characterized markers relating to stem cell function are integrins. β1 and α6 integrins are increased in keratinocytes in culture with high proliferative capacity, and they also appear to be enhanced in the bulge area, relative to the lower and upper segments of the anagen phase follicle. Interestingly, both β1 and α6 integrin mRNAs are among the 200 mRNAs identified in three different populations of stem cells when compared with their TA progeny, suggesting that the correlation between integrin levels and "stemness" may extend to stem cells beyond those of the skin.

The relationship between integrin levels and stem cells is intriguing, because it suggests the possibility that stem cells may be kept tight within the niche by adherence to each other and to the basement membrane. The need for stem cell progeny to migrate along the basement membrane surrounding the epidermis and its appendages may be fulfilled by a reduction in cell-substratum anchorage through down-regulation of integrins. Alternatively, however, it could be that, because cell proliferation is dependent on integrins, cells with high proliferative capacity by nature have elevated integrin levels. The extent to which regulation of integrins in the bulge is a reflection of hair growth mechanisms versus stem cell characteristics *per se* is an important issue, and one that has not yet been unequivocally resolved. Interestingly, up-regulation of cell migration associated integrin β6 in the ORS during anagen, when bulge and ORS cells migrate downwards to make the new hair follicle, seems to suggest the second possibility.

What cellular mechanisms regulate differences in integrin levels? Are there specific stem cell cues that prompt these changes in integrin levels in TA cells to allow them to exit the niche and migrate? These questions remain to be addressed. In the meantime, integrins are thought to play a major role in the skin, in general, regardless of stem cells in regulating epidermal adhesion, growth, and differentiation.

Other studies reported that proteins expressed differentially in the bulge. They include keratins K15 and K19, both of which are also present in a large fraction of basal layer cells; CD71 low, S100 proteins, E-cad low, p63, CD34, Sox9, NFATc1, Lhx2, and TCF3. By contrast, neonatal bulge cells express Sox9, NFATc1, Lhx2 and TCF3, but do not express CD34, nor are they active for the K15-promoter-lacZ, used to mark the adult bulge. Systematically testing the function of these and additional candidates that emerged from genomic screen adds new levels to our understanding of stem cell regulation at the molecular level.

CELL SIGNALING IN HAIR FOLLICLE STEM CELLS

What coaxes stem cells to become hair follicles rather than epidermis is one of the most fascinating questions in the skin stem cell field. Many of the answers are still not in, but hints have begun to emerge in the past five years. It has long been known that in embryonic development, as in postnatal hair cycling, this decision to coax cells toward the hair pathway involves critical cross-talk between the epithelial and mesenchymal cells. Interestingly, similar signals seem to be used in specification not only of hair, but also nail, mammary glands, and teeth.

In embryonic development of skin and its appendages, Wnt and bone morphogenic pathway (bmp) signaling have surfaced as two major players that are critical for normal morphogenesis. Gene targeting of Lef1, β-catenin sonic hedgehog, and the noggin inhibitor of the BMP pathway result in reduction, loss, or developmental impairment of hair follicles.

The Wnt signaling acts through a large family of soluble wnt morphogens that recognize a specific receptor family known as Frizzles to stimulate β-catenin, a dual protein that acts at the crossroads between cell adhesion and cell signaling. A Wnt signal results in an inhibition of β-catenin degradation, leading to an accumulation of the protein above and beyond that required for adherens junctions. β-catenin can then complex with members of the Lef/Tcf family of HMG DNA binding proteins and affect expression of transcription of downstream target genes. A truncated, constitutively active form of β-catenin expressed at high levels in the ORS of basal layer results in excess skin and *de novo* initiation of hair follicle buds in postnatal skin, a characteristic normally specific to the embryonic skin. Taken together, these data suggest that Wnt signaling may induce adult stem cells into a state more specific for their embryonic relatives, or that the Wnt signaling has a major role in specifying hair follicle fate to relatively undifferentiated cells.

In the adult hair follicle, two members of the Tcf/Lef1 family are expressed in strategic places. Lef1 is in the matrix, precortex, and DP, whereas Tcf3 is in the bulge, and the ORS of skin epithelium. mRNA-profiling revealed that Tcf3 mRNA is also up-regulated in bulge LRCs, suggesting that this regulation is at the level of gene expression. The localization of Tcf3 in the bulge is intriguing, because a related protein Tcf4 is found in the intestinal stem cell compartment, or crypt, where it appears to play a role in intestine stem cell maintenance. *In vitro*, Tcf3 acts as a repressor in the absence of Wnt signaling, and, in its presence, it can be converted to an activator. *In vivo*, transgenic expression of the repressor forms of Tcf3, both in stem cells and TA cells of the skin, results in a lethal phenotype, in which epidermal basal cells are conferred ORS characteristics. In contrast, overexpression of TCF3 in the adult skin leads to repression of all epithelial lineages. In contrast to Tcf3, Lef1 is expressed in the matrix, but accumulates more strongly in the nuclei of precortical cells. The progenitor cells of the hair shaft precortex express a bank of hair-specific keratin genes that possess Lef1 binding

sites in their promoters. Precortex also shows nuclear β-catenin, and it expresses a Wnt-responsive reporter gene TOPGAL. These cells are thus likely to receive a Wnt signal, and Wnt expression is seen in this region of the anagen hair follicle. Interestingly, when expressed in hair precursor cells in transgenic mice, dominant negative forms of Lef1 result in the production of SG cells, while loss of β-catenin results in production of epidermal cells at the expense of hair differentiation. In contrast, the transcription factor GATA3 is known to be indispensable for inner root sheath differentiation. Curiously, both Lef1 and GATA3 have been implicated in hematopoietic stem cell lineage determination.

Although Wnts are clearly necessary for β-catenin stabilization, to obtain a Wnt response, a cell must express Tcf/Lef factors. Jamora *et al.* showed that noggin, a BMP inhibitor, can induce Lef1 expression in keratinocytes *in vitro*, a feature corroborated in keratinocytes and skin epithelium null for the BMP receptor-1a. When these cells are also treated with Wnt3a, they affect the transcription of Wnt-responsive reporter genes. In most cases, this combination leads to transactivation, but curiously, the E-cadherin promoter seems to be down-regulated by these factors. *In vivo*, down-regulation of the E-cadherin promoter accompanies induction of hair placodes in embryogenesis and secondary hair germs in cycling hair follicles, and transgenic elevation of E-cadherin blocks follicle morphogenesis. Conditional ablation of the Bmpr1a gene results in activation of the quiescent SCs to proliferate, causing an expansion of the niche and loss of slow-cycling cells. The HFSCs generate long-lived, tumor-like branches that display elevated stabilized β-catenin and express Sox9, Lhx2, and Sonic hedgehog, but fail to terminally differentiate to make hair. An important aspect of the BMP regulation of HFSCs resides in skin compartments other than the bulge itself. This stem-cell extrinsic function has been identified within the mesenchymal pocket of dermal papillae cells. When the BMPR1a receptor is deleted in these cells, they cannot receive BMP signals, and lose their well-established hair follicle inductive properties. Moreover, BMP is important in the dermal cells surrounding the bulge, where it regulates the cyclic activation of bulge stem cells in traveling waves regionally controlled across the mouse body.

Taken together, these findings suggest a model whereby activation of Wnt signaling and inhibition of BMP signaling work together to maintain Wnt responsiveness in skin epithelial cells and show that, through down-regulation of E-cadherin, the cells are able to undergo epithelial cell–cell remodeling, necessary for follicle morphogenesis. Moreover, the findings suggest that adult and embryonic stem cells may use similar signals to control their differentiation.

An intriguing feature of the Wnt signaling pathway is that when constitutively activated, it leads to elevated levels of tissue progenitor cells in skin, in brain, and in intestine. In addition, purified Wnts stimulate isolated hematopoietic stem cells to proliferate in culture, and following skeletal muscle injury, Wnts appear to mobilize resident stem cells during the regeneration process. Taken together, these findings implicate the Wnt signaling pathway in the self-renewal of stem cells and/or their transit-amplifying progeny. Consistent with these observations is the recent finding that the growth-restricted environment of the bulge is associated with an up-regulation of mRNAs more typically affiliated with Wnt-inhibition. Future studies will be required to assess whether this tantalizing correlation is functionally significant.

In the most recent years, a series of developmentally-controlled transcription factors, such as LHX2, NFATc1, RUNX1, and c-MYC, have surfaced as positive or negative regulators of hair follicle stem cell proliferation during the quiescence and activation cycles of normal adult hair homeostasis. Deletion of another transcription factor, Sox9, resulted in alopecia with perturbed maintenance of HFSCs. Reduced numbers of CD34+ cells were detected in VDR knockout mice, a defect that could be rescued by inducible transgenic activation of β-catenin. In a different category of hair follicle stem cell regulatory factors lies the Rho GTPase Rac1; its deletion resulted in conversion of slow-cycling cells to more frequently dividing, presumably TA cells, and eventually loss of the stem cell pool. Intriguingly, when Rac1 was constitutively deleted in the skin epithelium, the phenotype was not detected, potentially due to compensatory mechanisms activated through development. Furthermore, an important aspect of stem cell regulation seems to take place through telomerase activity. This activity relies on both regulation of telomere length to prevent stem cell replicative senescence, which is dependent upon the RNA component of the complex (TERC), and on a telomere-length independent function. The later has implicated the protein component of telomerase (TERT) in activation of HFSCs from the quiescent phase, through transcriptional regulation of MYC and Wnt responsive genes.

Integration of these and additional factors in complex networks of gene activity remains one of the tantalizing future challenges of the field. As these networks consolidate, it will become apparent how misregulation of one pathway could affect the entire system's function, and how subtle perturbation of any node in the network could be amplified to lead to disease.

COMMENTARY AND FUTURE DIRECTIONS

Probing more deeply into the molecular mechanisms of stem cell maintenance and lineage determination in the skin is now possible using newly-identified factors that distinguish putative bulge stem cells from their progeny.

Interestingly, a large fraction of these factors are either secreted or transmembrane factors that are likely involved in interactions with the environment. Some of these have been previously associated with stimulation of different cell types that coincidentally surround the bulge area, whereas others are likely involved in extracellular matrix and basement membrane organization. Still others are candidates for creating a local gradient of signaling molecules that could maintain stem cells within the bulge area in a growth- and differentiation-inhibited environment. These findings are in agreement with the bulge being an entity with tissue morphogenesis activity upon grafting, and begin to unravel the molecular mechanisms and the identity of the cells within the bulge that perform this activity. If future tests of these candidates support these predictions, then the concept of preformed niches, in which stem cells find their "home," may have to be reconsidered. In this regard, somatic stem cells may be even more potent than initially surmised, and may be actively involved in building and maintaining their own niches through adult life.

For skin stem cells in particular, many of the most interesting questions still remain unaddressed. What specific genes are responsible for stem cell maintenance in an undifferentiated state, and for their self-renewal? How will their expression change during the hair cycle? How will their patterns change as stem cells either exit from their niche and migrate *in vivo*, or are removed from their niche and cultured *in vitro*? How does wounding influence the status of stem cells, and what particular signals penetrate the bulge to instruct cells to exit and help replenish the epidermis? How strong is the correlation between slow-cycling cells and stem cells? Are there long-term and short-term stem cells, and, if so, what makes them different? Do hair follicle stem cells of the skin undergo asymmetric or symmetric divisions?

As we begin to learn more about the stem cells of the skin, and the cues that these cells respond to, a clear picture should emerge as to how stem cells are coaxed along specific lineages, and how their niche impacts on this decision-making process. As we gain further insights into these issues, and explore in more depth the relationship between adult and embryonic skin stem cells, and the parallels between skin stem cells and other stem cells of the body, including pluripotent embryonic stem cells. It is the ultimate goal of the stem cell field to understand enough about the various unipotent, multipotent, and pluripotent cells of the body to make major inroads in the interface between stem cell biology and human medicine.

KEY WORDS

Hair cycle Succession of growth, regression, and rest that the hair follicle undergoes during the life of an animal.

Label retaining cells Cells that incorporate labeled nucleotides during the S phase and retain it preferentially over extended periods of time due to slow or infrequent divisions.

Stem cell activation Process that stimulates stem cells to exit their dormant, quiescent, undifferentiated state, and begin to produce more committed cells to participate in tissue growth or repair.

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Chapter 23

The Ontogeny of the Hematopoietic System

Malcolm A. S. Moore

HISTORIC PERSPECTIVE

The origin and potentiality of the hematopoietic stem cell has been debated for over a hundred years, with two main concepts: the monophyletic hypothesis recognizing a common stem cell for all lympho-myeloid lineages; and the polyphyletic hypothesis recognizing a variety of distinct stem cells. The current consensus recognizes a pluripotential lympho-myeloid stem cell, and a hierarchy of progressively lineage-restricted progenitor cells with a major bifurcation at the level of the common lymphoid and common myeloid progenitor. One early assumption had been that each developing hematopoietic or lymphoid organ produces its own complement of stem cells, with morphological studies variously proposing an *in situ* origin in hematopoietic tissues from undifferentiated mesenchyme or endothelium and in primary lymphoid organs, such as thymus or avian bursa of Fabricius, a derivation from epithelium. Over 35 years ago, this view was challenged in studies in avian embryos that showed developing hematopoietic and lymphoid organs were colonized from the outset by an inflow of stem cells from the bloodstream. Since the yolk sac (YS) was identified at that time as the first site of hematopoiesis, it was proposed as the site of origin of stem cells that colonized all subsequent hematopoietic and lymphoid organs. This hypothesis was challenged by later studies that demonstrated an embryonic origin of hematopoietic or lymphoid cells that later appeared in spleen, marrow, thymus, and bursa of Fabricius.

In mammalian systems, hematopoiesis is clearly initiated in the YS, and subsequently in the aorta–gonad–mesonephros (AGM) region in association with the dorsal aorta and vitelline veins where the first stem cells arise with the capacity to engraft adult recipients. There has been extensive debate as to the role of the YS in mammalian hematopoietic ontogeny with, on the one hand, the site viewed as the initial source of stem cells that then migrated into the embryo, and, on the other hand, the other extreme that views the YS as a transient source of primitive erythroid progenitors, with definitive generation stem cells arising exclusively in the AGM region. There is increasing evidence that stem cells arise independently in both sites, and that both contribute to the subsequent colonization of the fetal liver. Within the last two years, a number of studies have revived the concept of a YS origin of intraembryonic hematopoiesis in murine development; however, the placenta has also been implicated as a *de novo* site of HSC development.

SITES OF INITIATION OF PRIMITIVE AND DEFINITIVE HEMATOPOIESIS AND VASCULOGENESIS

The sites of hematopoiesis, and the primary sites of lymphopoiesis, with times of initiation of activity in development, are shown in Table 23-1.

TABLE 23-1 Comparative Chronology of Hematopoietic Development

Initiation of Hematopoiesis or Lymphopoiesis (days)	Mouse	Human	Chicken
Yolk sac	7.5	18	2
Aortic/AGM region	9.5–10	27	3
Liver	11	42	No
Spleen	13	48	8
Bone marrow	15	77	11
Thymus	11	40	7
Bursa of Fabricius	No	No	14
Onset of circulation	8.2	24	2.5

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Yolk Sac Development

The YS forms during gastrulation, which begins in the mouse embryo at day 6.5 (E6.5). Mesodermal cells destined for extraembryonic sites exit the posterior primitive streak and subdivide the embryo into three separate cavities by the neural plate stage (E7.5). The central cavity, the exocoelom, becomes completely lined with mesoderm and, where this is adjacent to visceral endoderm, visceral YS forms. Between E7 and E7.5, mesodermal cells in the visceral YS proliferate and form mesodermal cell masses that are the precursor of the blood islands. Central cells accumulate hemoglobin, while the outer cells flatten and form endothelium. Lineage-tracing experiments show that the hematopoietic mesoderm arises from posterior primitive streak mesoderm. Tissue recombination studies in the chick embryo also indicated that YS hematopoiesis requires diffusible signals from extraembryonic endoderm (hypoblast) that is analogous to visceral endoderm of the mouse. Indian hedgehog (Ihh) and smoothened (Smo), a receptor component essential for all hedgehog signaling, are required for yolk sac development. Ihh appears to be the endodermal signal inducing hematopoietic and vascular specification of YS mesoderm, acting via induction of bone morphogenic protein-4 (BMP-4) in mesoderm, that in turn induces hemato-vascular development (Figure 23-1).

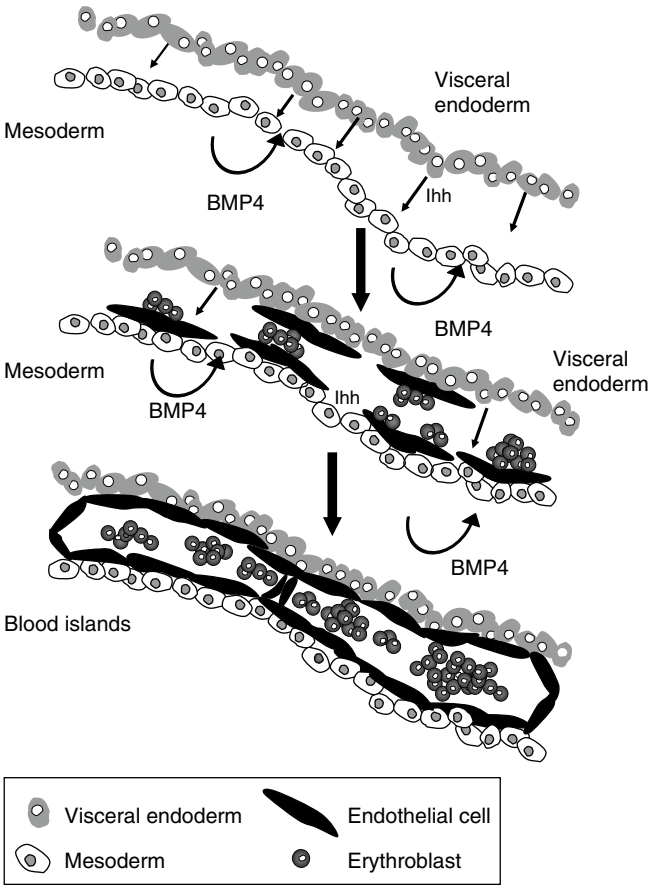


FIGURE 23-1 Activation of primitive hematopoiesis and vasculogenesis in the developing yolk sac. Indian hedgehog (*Ihh*) is secreted from visceral endoderm to the target extraembryonic (yolk sac) mesoderm, where it activates expression of *bmp4*. BMP4 protein in turn feeds back to extraembryonic mesoderm (in an autocrine or paracrine manner), activating genes such as *Flk1*, *CD34*, *SCL/tal-1*, and *AML1*. This is associated with formation of hematopoietic and vasculature stem and progenitor cells, possibly via a common precursor, the hemangioblast. Reproduced with permission from Baron, M. H. (2001). *J. Hematotherapy & Stem Cell Research*, *10*, 587–594.

Onset of Primitive and Definitive Hematopoiesis in the Yolk Sac

The YS is the site of both primitive erythropoiesis and macrophage production, and also of definitive multi-lineage, and myeloid and erythroid lineage-restricted, progenitor cell production. The wave of primitive erythropoiesis begins in the YS at E7.5, with nucleated red cells producing embryonic globin predominating in the circulation through E14.5, and with eventual replacement by fetal liver-derived red blood cells (RBC) expressing adult globins by E15.5–16.6. In humans, β-like embryonic globin (hemoglobin ε) is expressed first in embryonic nucleated RBCs in YS blood islands. Subsequently, fetal globins (hemoglobin Ag and Gg) are expressed in definitive RBCs developing in the fetal liver. Finally, adult δ- and β-globins are expressed around the time of birth within bone marrow-derived RBCs. The zinc finger transcription factor, Erythroid Kruppel-like Factor (EKLF), plays a role in coordinating erythroid cell proliferation and hemoglobinization, participating in the switch from embryonic to fetal, or fetal to adult β-globin expression.

A population of primitive high-proliferative potential progenitors colony forming cells (HPP-CFC) were detected at E8 (1–8 somites) in the mouse embryo, exclusively in the YS. This remained the predominant site of expansion (>100-fold) of these multipotent precursors until E10.5–11.5, when there was a dramatic increase of HPP-CFC in the circulation and liver, with a concomitant drop in the YS. Upon secondary replating, these HPP-CFCs exhibited the stem cell features of self-renewal and potential to generate definitive erythroid and macrophage progeny. Separation of YS and embryo at the early E8 stage, before a common circulation is established, showed that HPPs were found exclusively in the YS, and examination of the blood at E8.5 suggested that they entered the circulation from YS together with the first erythroblasts. Definitive erythroid (BFU-E) and myeloid (CFU-GM) progenitors and mast cell precursors are detected in the YS at E8, but do not differentiate there; rather, they migrate to the fetal liver where BFU-E/CFU-E initiate definitive erythropoiesis.

The YS does not contain cells capable of long-term multi-lineage engraftment of adult irradiated mice until after such cells have appeared in the AGM region at E10.5. Although it has been argued that the AGM region is the

sole site of origin of "definitive" HSC capable of engrafting adult recipients, and the presence of these cells in the YS at later stages represents an immigrant population, total body quantitation and tissue distribution analysis of competitive repopulating HSC detected by limiting dilution analysis argues for two independent sources of "definitive" HSC, both participating in seeding the fetal liver.

Support for this view was obtained in co-culture studies of either E8–8.5 YS or intraembryonic splanchnopleuric mesoderm (P-Sp) on a stromal cell line generated from the AGM region. The conventional definition of an HSC is that it must possess extensive self-renewal potential and multilineage differentiation potential. By this criteria, the YS contains HSC as early as E9, as demonstrated in a number of studies showing that CD34+, c-Kit+, and CD38+ YS cells at this stage, when injected directly into the liver of busulphan-conditioned neonatal mice, gave long-term lympho-myeloid reconstitution. Secondary engraftment in sublethally irradiated mice demonstrated that the YS-derived HSC acquired normal marrow homing properties.

The argument that the "true" stem cells must have the ability to repopulate the adult mouse after intravenous injection is not relevant to normal ontogeny, since the only requirement of the initial HSC population is that it be capable of colonizing the liver. Within that environment it may then acquire marrow homing features that permit it to become an "adult" stem cell. The molecular basis for the distinction between the embryonic and adult-type stem cell is unknown, but may involve adhesion receptors, including CD34, CD44, selectins, and α- and β-integrins. In this context, β1-integrin-deficient HSC fail to seed both fetal and adult hematopoietic organs.

The CXCR4 receptor and its ligand, the chemokine SDF-1, mediate HSC chemotaxis and are critical for HSC homing to marrow, but not for fetal liver colonization. Co-culture of YS on AGM stroma confers adult engraftment potential, suggesting an inducing role for stromal cells in this region, particularly mesenchymal cells on the floor of the dorsal aorta. As currently envisaged, this inductive influence confers HSC potential in locally-generated hematopoietic clusters. However, it is conceivable that early YS HSC traffic into the dorsal aorta and come under the influence of the AGM stroma, thus acquiring adult engraftment potential.

Three distinct views of early mouse hematopoiesis have evolved. As initially proposed, the YS blood islands were identified as the site of *de novo* generation of hematopoietic cells from mesodermal precursors, including the HSCs that seed the fetal liver. A more physiologically relevant way to address whether or not the YS contributes to adult hematopoiesis is by genetically labeling YS cells at their early stages before the establishment of circulation, and measuring their contribution to later stages of hematopoiesis *in vivo*. A recent study by Nishikawa and his group utilized the Cre/lox recombination system to ask this question. A gene encoding a fusion protein containing the Cre recombinase gene flanked by two copies of a mutated estrogen receptor was placed under the transcriptional control of the Runx-1 locus, which is selectively expressed in the YS of the pre-circulation embryo. Nuclear localization of Cre was transiently induced by injection of tamoxifen, resulting in the activation of reporter gene expression in the Runx-1-expressing YS cells and their progenies. By following the labeled cells during embryonic development and adult stages, the authors concluded that some YS-derived cells migrate through AGM, umbilical cord, and FL, seed the BM, and contribute to adult hematopoiesis. Non-invasive cell-tracing analysis demonstrated that YS blood island-derived precursors migrated to the luminal surface of vessels in the embryo, umbilical cord, fetal liver, and thymus. Thus, YS-derived pre-HSCs acquire functional HSC characteristics in the AGM/umbilical artery and umbilical vein region.

The relative contribution of YS derived cells to the circulating definitive HSC pool that seeds the fetal liver remains controversial, due to the presence of systemic circulation and the onset of hematopoiesis within the embryo proper prior to liver seeding. To test this, Yoder and his group studied mice with deletion of Ncx-1. Ncx-1$-/-$ embryos failed to initiate a heartbeat on embryonic day (E) 8.25, with interruption of the onset of circulation and failure in the development of intra-embryonic hematopoiesis, while YS definitive hematopoiesis was quantitatively normal. Primitive erythroblasts and essentially all definitive HSC/HPC destined to initially seed the fetal liver after E9.5 are generated in the YS between E7.0–E9.5, and are redistributed into the embryo via the systemic circulation. In another study, Rac-1 was deleted during very early embryonic development, and deficient E10.5 embryos showed a decrease in circulating HSC/HPC while YS definitive hematopoiesis was quantitatively normal. Intra-embryonic hematopoiesis was significantly impaired, culminating in the absence of intra-aortic hematopoietic clusters and fetal liver hematopoiesis. These data suggest that Rac-1 plays an important role in HSC migration during embryonic development, and is essential for the emergence of intra-embryonic hematopoiesis. These targeted deletion models suggested that the YS is a predominant source of HSC, and is responsible for seeding all subsequent sites of lympho-hematopoiesis. However, the placenta, not previously recognized as a significant site of embryonic hematopoiesis has recently been shown to be a *de novo* site of HSC development, as demonstrated in Ncx1$-/-$ mice. The relative contributions of the YS and placenta to subsequent lympho-hematopoietic development remain to be elucidated.

Macrophage and Microglial Ontogeny

In murine ontogeny, primitive macrophage progenitors appear in the proximal region of the egg cylinder associated

with expression of SCL/tal-1 and GATA-1 at E7. They increase until the early somite stage (E8.25), and then they decline sharply to undetectable levels by the 20 somite stage. Definitive lineage multipotent precursors with macrophage potential (HPP-CFC), as well as more restricted definitive CFU-GM and CFU-M, appear at E8.25. Separation of YS and embryo at 8.25 (before the circulation) showed that HPP-CFC was found exclusively in YS. Once the circulation is established, HPP-CFC can be found in the circulation, indicating migration into the embryo.

In avian studies, chick-quail chimeras established as early as E2.5 show numerous YS-derived cells, including macrophages, within the embryo vasculature and within mesenchyme. At late E2, numerous scattered CD45+ cells appear in YS and in the blood, exiting from the circulation through the endothelium and rapidly invading the whole embryo. These cells express high levels of CD45 and correspond exactly to cells identified as monocyte–macrophages. YS-derived macrophages showing marked acid phosphatase activity and phagocytic capacity appear within the neural tube, liver anlage, and nephric rudiments, beginning even before the circulation is established. In the mouse, the macrophage progenitors appearing in the YS at E7.5–8 migrate into the mesenchyme surrounding the brain rudiment. Initial migration occurs before a circulation is established and is interstitial, but after E8.2 seeding occurs via the vasculature. These YS-derived macrophages continue to proliferate and generate the brain microglia, which ultimately comprises 10% of the brain. The primitive generation YS-derived macrophages develop at E7–8 in the absence of a monocyte or promonocyte intermediary stage, and differ from adult macrophages in the pattern of enzymes produced. Peroxidase-positive promonocytes of the definitive lineage appear in YS at E10.

Ontogeny of the Vasculature

Morphogenesis of blood vessels is defined by a sequential pattern of gene expression in which SCL/Tal1 and Flk1 are expressed first at the angioblast stage, followed by PECAM, CD34, VE-cadherin, and later Tie2, while SCL expression is down-regulated in endothelium of mature vessels. As somatogenesis begins, vascular development is asymmetric, centered in YS blood islands, and in the embryo proper where angioblasts begin to coalesce into the aorta. By the murine 3 somite stage, vascular development has spread through the YS and extended aortic tubes are visible. Beginning at the 4 somite stage, erythroblasts disperse through the distal yolk sac, and a few erythroblasts appear in the head and tail region of the embryo where the embryonic and YS vasculature meet. The circulation onset is defined as erythroblast movement within vessels, and is initiated at the murine 4–6 somite stage during E8.25.

Initiation of Hematopoiesis in the Aorta-gonad Mesonephros Region

Avian Intra-aortic and Para-aortic Hematopoiesis

In birds, the first intraembryonic hematopoiesis occurs at E3 in endothelium-associated intra-aortic clusters of markedly basophilic cells with prominent nucleoli, and subsequently (E6–8) in diffuse hematopoietic "para-aortic foci" located in the dorsal mesentery ventral to the aorta. Hematopoietic progenitors are detected in the aortic wall at E3–4. At E2, prior to the appearance of the intra-aortic clusters of CD45+VEGFR-2−ve hematopoietic cells, the aortic endothelium consisted entirely of CD45−ve, VEGFR-2+ flat endothelial cells. Chick-quail marker studies showed that the intra-aortic clusters developed *in situ*, and not by migration from the YS. Acetylated low-density lipoprotein (Dil-Ac-LDL) labeling indicated that the hematopoietic clusters originated from aortic endothelium, while retroviral vector marking indicated that the para-aortic foci were derived from intra-aortic clusters. Mesodermal subdivision transplantation showed that the endothelium of the roof and sides of the dorsal aorta arose from somite mesoderm that generates "pure" angioblasts, while the floor develops from splanchnopleural mesoderm that generates progenitors with dual hematopoietic and angiogenic potential.

In the human embryo, hematopoietic clusters containing CD34+ cells appear in the aorta at 27 days gestation, eight to nine days after initation of YS hematopoiesis and six days after the onset of the circulation (Figure 23-2). The precursors of the hematopoietic clusters in the periaortic region were considered "endothelial" based on expression of CD34 and CD31 (PECAM-1), absence of CD45, and uptake of Dil-Ac-LDL. In mice, hematopoietic clusters appear at E10.5 in the ventral floor of the dorsal aorta. They appear to be in direct contact with underlying mesenchyme where the endothelium is interrupted. However, ultrastructural studies in humans show that the endothelial basal lamina is intact where it interacts with the cells in the hematopoietic clusters, and the hematopoietic and endothelial cells are interconnected by tight junctions (Figure 23-2). VE-cadherin-expressing cells in the AGM have hematopoietic potential, and VE-cadherin is expressed on the luminal aspect of vascular endothelium, but not in CD45+ aorta-associated hematopoietic clusters. VE-cadherin is essential in stabilizing tight junctions between endothelial cells, suggesting the endothelial origin of hematopoiesis. Similar populations of CD34+CD45−ve cells in the human para-aortic region are labeled with the endothelial marker *Ulex Europus*, and generate both von Willebrand (vW)+ endothelium and hematopoietic cells. This blood-forming "endothelium" is present in the human embryo AGM region at 28 days, with a peak frequency at 31 days and absence by 44 days. Culture of the paraortic splanchnopleura (P-Sp) from 21- to 26-day

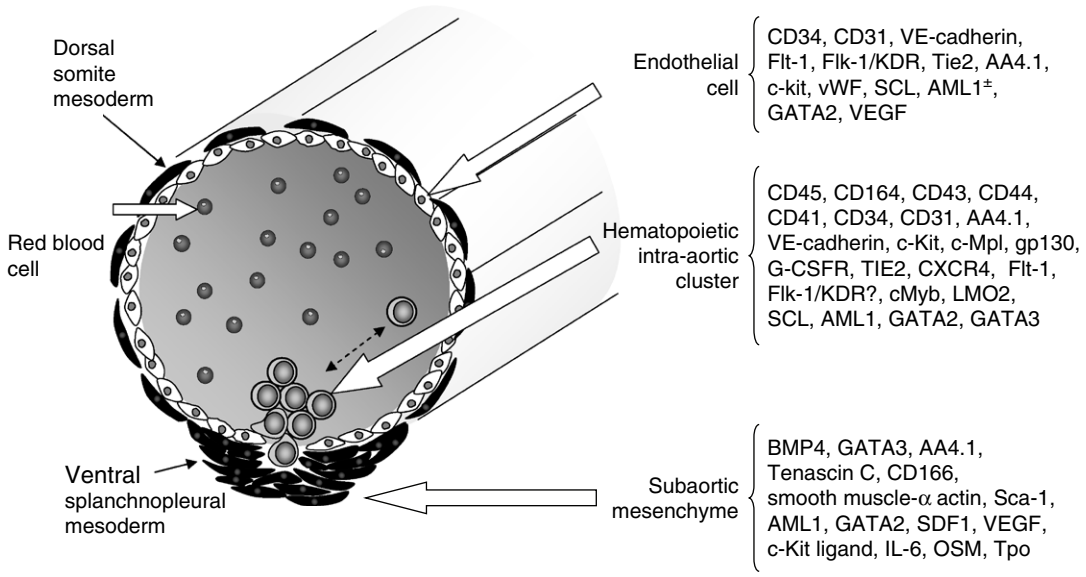


FIGURE 23-2 Intra-aortic hematopoietic cluster formation in the floor of the dorsal aorta, based on data from E10.5 murine and E27 human studies. The dorsal endothelium of the aorta derived from angioblasts develops from somite mesoderm, whereas the ventral "hemogenic endothelium," or hemangioblast, develops from splanchnopleural mesoderm. The dorsal endothelium expresses markers associated with differentiated endothelium. The hematopoietic intra-aortic cluster expresses surface antigens and cytoadhesion molecules found on hematopoietic stem and progenitor cells, hematopoiesis-related transcription factors, and receptors for hematopoietic and angiogenic growth factors. The expression of Flk-1 on intra-aortic clusters is controversial. The subaortic mesenchyme expresses cytokines, chemokines, and cytoadhesion molecules, and has molecular features of vascular smooth muscle.

human embryos on marrow stromal cells generated hematopoietic cells, demonstrating the hematogenic potential of P-Sp mesoderm that precedes the appearance of "hemogenic endothelium." The pre "endothelial" mesodermal precursor is CD34−ve, CD45−ve, and VEGFR-2/KDR+.

Since cells present in the human embryo AGM prior to 28 days lacked direct hematopoietic potential, it was postulated that they must receive some inductive signal from surrounding tissue to induce this potential. A discrete region of densely packed mesenchymal cells lies beneath the ventral floor of the dorsal aorta and is three to four layers thick in mouse and five to seven layers thick in humans (Figure 23-2). These mesenchymal cells are interconnected by tight junctions, express smooth muscle α-actin (SMα-A), and may be precursors of vascular smooth muscle. BMP-4 may play a crucial role in the induction of hematopoietic potential in the AGM region, much as it does in the YS. BMP-4 is polarized to the ventral wall of the dorsal aorta in the mesenchymal layer underlying the intra-aortic hematopoietic clusters, where it could induce P-Sp mesoderm to become hemangioblasts and HSC (Figure 23-2).

The mesenchymal inductive influence extends beyond the induction of multi-lineage hematopoietic precursors, and potentially includes induction of development of B and T lineage-restricted progenitors, and of HSC capable of long-term repopulation of adult mice. The mouse AGM region at E10.5 was identified as a source of CFU-S, preceding their appearance in YS. CFU-S precursors were present in both AGM and YS by E9, since cultures of either tissue at this

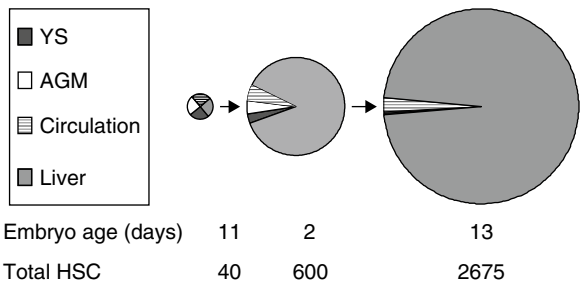


FIGURE 23-3 Origin and distribution of definitive hematopoietic stem cells (numbers of competitive repopulating units (CRU) detected by limiting dilution competitive long-term repopulation assay in adult irradiated mice, adjusted for a calculated seeding efficiency of 10%). The figure shows absolute numbers in each tissue at daily intervals from E11 to E13 in the mouse embryo. Modified after Kumaravelu, P., *et al.* (2002). *Development*, *129*, 4891–4899.

stage generated CFU-S. *In vivo* engraftment was obtained with cells from the E10.5 dorsal aorta and vitelline/umbilical artery. Quantitation of long-term repopulating stem cell numbers by limiting dilution assay has shown that they first appear in the AGM at E10.5, and are present in equal numbers in AGM, YS, circulation, and early liver rudiment by E11 (Figure 23-3). The colonization of the fetal liver by stem cells is initated by a wave of migration from the AGM peaking at E11, followed by a second wave from the YS (Figure 23-4 and 23-5). Cells capable of long-term lympho-myeloid engraftment have been reported to be generated in organ culture of P-Sp isolated prior to the onset of the circulation

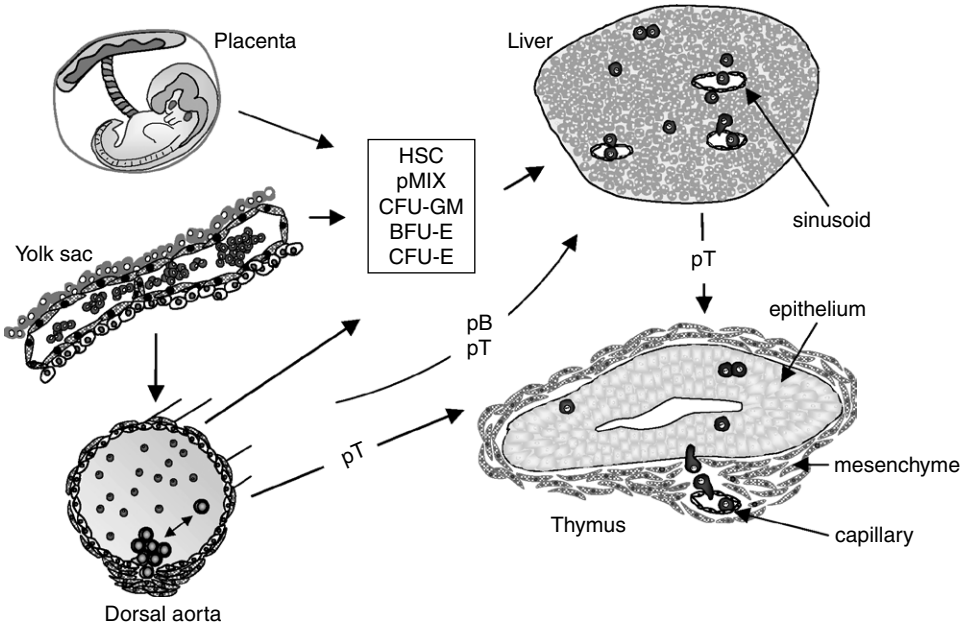


FIGURE 23-4 Vascular migration streams of hematopoietic stem cells (HSC) and myeloid (CFU-GM, BFU-E, CFU-E, pMIX) and lymphoid (pT, pB) progenitor cells between the dorsal aorta/AGM, the yolk sac blood islands, the placenta, the embryonic liver, and the thymus, occurring between E10.5 and E11.5 in the mouse embryo. Immigrant cells (shown as basophilic blast cells) detach from the YS blood islands and from the aortic hematopoietic clusters, enter the circulation, and subsequently egress from the microvessels within the hepatic rudiment, or within the perithymic mesenchyme, and accumulate and proliferate within these developing organs.

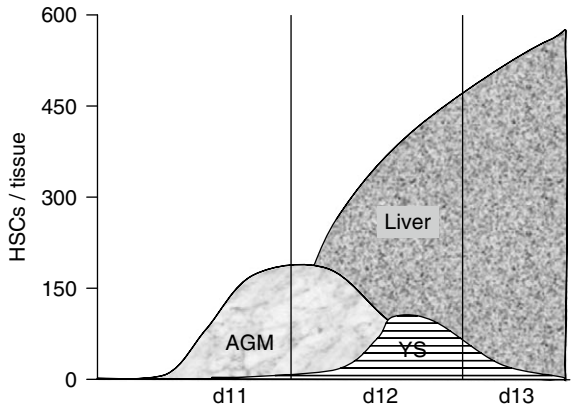


FIGURE 23-5 Schematic representation of colonization of the embryonic liver of the mouse embryo with HSC, as determined by CRU assay in adult irradiated mice and adjusted for seeding efficiency. The numbers are based on the numbers that the AGM and YS are able to generate *in vitro*. *In vivo*, the high cumulative activity of the AGM region and the YS may provide the liver with a high proportion of definitive HSC. The data suggests consecutive colonization of the embryonic liver with HSC from the AGM region and the YS. Reproduced with permission from Kumaravelu, P., *et al.* (2002). *Development*, *129*, 4891–4899.

(E7.5–8), whereas cultured YS failed to generate such HSC. However, other investigators were able to generate repopulating HSC in co-culture of equally early P-Sp and YS, with a stromal cell line derived from the E10.5 AGM region.

The various inductive influences of the subaortic mesenchyme could cause bipotential precursors located in the floor of the aorta to preferentially adopt a hematopoietic fate. Alternatively, endothelial cells may dedifferentiate and switch to a hematopoietic fate in response to local or transient signals. A third possibility is that hemangioblasts or their precursors may migrate secondarily into the aortic wall, either from the underlying mesenchyme or from the circulation, in response to a chemokine gradient. Endothelial cells, angioblasts, and HSC express CXCR4, and mesenchyme adjacent to the dorsal aorta expresses high levels of its ligand SDF-1, indicating a role for this chemokine pathway in both vasculogenesis and migration of hemangioblastic precursors to the floor of the aorta.

Studies using embryonic stem (ES) cells differentiating for 2.5 to 3.5 days have demonstrated the transient development of cells capable of forming blast cell colonies (BL-CFC) in the presence of VEGF and the c-Kit ligand/stem cell factor (SCF) that precede the appearance of hematopoietic colony-forming cells. Cells in the blast colony express a number of genes common to hematopoietic and endothelial lineages (CD34, SCL, Flk-1), and on replating give rise to endothelial progenitors and both definitive and primitive hematopoietic progenitors. The thromobopoietin (Tpo) receptor c-Mpl was detected by day three of embryoid body formation, when hemangioblasts first arise. Tpo alone supported BL-CFC formation, and nearly doubled the number of BL-CFC when combined with VEGF and SCF. Since hematopoietic and endothelial development are not extinguished by targeted inactivation of *c-Mpl* or *Tpo* genes, there must be some redundancy of cytokine pathways acting on hemangioblasts. Other overlapping pathways active on hemangioblasts include VEGF/Flk-1 c-Kit/SCF, BMP-4, and TGFβ. The ES results support the concept of a hemangioblast as the precursor of both endothelium and hematopoiesis. Tpo and c-Mpl transcripts are present in the early YS prior to appearance of the first blood islands, and c-Mpl is expressed on embryonic blood vessels and aorta. Other cytokines stimulating hemangioblasts are expressed in the AGM region, including VEGF, SCF, and BMP-4.

The respective roles of hemangioblast and "hemogenic endothelium" for initiating hematopoiesis in the AGM or YS is debated. A number of these studies identify endothelium as the source of hematopoiesis, based on isolation of cells expressing markers associated with endothelium (VE-cadherin, CD31/PECAM, Flk-1, Flt-1, Tie-2, *Ulex Europus* binding, Dil-Ac-LDL uptake). None of these markers is unique to endothelium, and possibly could be expressed on hemangioblasts or prehematopoietic mesoderm, whereas some are expressed on HSC. Direct derivation of hematopoietic cells from mature, already differentiated, endothelium has been reported in Ac-LDL-DiI labeling studies. Molecular analysis of single cKit+CD34+ cells from the E11 mouse AGM region, when populations of engraftable HSC as well as lymphoid and myeloid committed progenitors are present, showed that the majority expressed hematopoietic-specific transcription factors AML-1, GATA-2, PU-1, and LMO2. The majority of cells also express the G-CSF receptor, while a minority express the erythropoietin receptor. The myeloid-specific gene MPO was expressed in 90% of cells, while the erythroid specific β-globin was expressed in 50%, indicating that genes of mutually exclusive differentiation lineages may be expressed simultaneously in a single primitive cell, prior to lineage commitment.

AML1 plays a critical role in establishing definitive hematopoiesis in the AGM. AML1 expression is initiated in mesenchymal cells at the distal tip of the allantois, and endothelial cells in the ventral portion of paired dorsal aorta at E8.5, and in both endothelium and mesenchyme of the ventral AGM and in the intra-aortic hematopoietic clusters between E9.5–11.5. Up to a third of the cells recovered from the AGM and the vitelline and umbilical arteries at E11.5 were AML1+, and this population contained all HSC capable of engrafting irradiated recipients. These HSC were initially CD45−ve at E10.5, but by E11.5, HSC activity was present in both CD45+ and CD45−ve fractions.

In AML1-deficient mice, HSC and intra-aortic clusters were absent, whereas in AML1 hemizygous mice, hematopoietic clusters were reduced in number and size and HSC numbers and distribution were altered, indicating a gene dosage effect. Haploinsufficiency of AML1 results in an earlier appearance of engraftable HSC in the YS with E10 YS cells reconstituting the hematopoietic system of irradiated adult mice. At the same time, there is premature termination of HSC activity in the AGM explants, consistent with a change in the balance of HSC emergence, migration, and/or maintenance. With hemozygous levels of AML1, the YS may autonomously generate HSC simultaneous to their generation in the AGM, or AGM may autonomously generate abundant HSC that immediately and rapidly migrate to the YS where they are detected in abundance, or AML1 insufficiency blocks emigration of HSC from the YS resulting in accumulation there and a deficiency in the AGM.

STEM CELL MIGRATION TO LATER SITES OF HEMATOPOIESIS

Ontogeny of Liver Hematopoiesis

Lineage-tracing studies support the derivation of the liver from ventral foregut endoderm that is induced by signals from pericardium and septum transversum mesenchyme, to proliferate and adopt a hepatic state. The fetal liver stroma consists of cells that express features of epithelium (cytokeratin-8), mesenchyme (vimentin, osteospondin), and vascular smooth muscle (α-smooth muscle actin). These cells are supportive of long-term HSC proliferation, and their presence in the liver coincides with the duration of hepatic hematopoiesis. At late gestation, when hematopoiesis declines, these cells are replaced by epithelial cells resembling mature hepatocytes and a minority of myofibroblasts. Oncostatin M, produced by hematopoietic cells within the liver, has been implicated in inducing hepatic maturation of the epithelial-mesenchymal stromal cells with loss of hematopoietic support capacity.

Fetal liver isolated before the 28 somite stage (E9.5), and grafted beneath the kidney capsule of adult mice, resulted in survival of hepatic tissue, but no hematopoietic elements were present. Administration of hematopoietic cells into the circulation of recipient mice resulted in multilineage hematopoietic engraftment of the implanted fetal liver. Grafts of liver isolated at >28 somite stage showed autonomous hematopoiesis, defining the time of initiation of HSC entry into the liver at E9.5–10. In humans the number of BFU-E drops abruptly in the YS at 35 days, and simultaneously they appear in the liver, reflecting oriented migration. The definitive BFU-E and CFU-E that are generated in the YS do not differentiate in that site, and most likely seed the fetal liver and rapidly establish definitive erythropoiesis. This is supported by the observation that large numbers of CFU-E appear simultaneously with BFU-E at the onset of hepatic hematopoiesis, and embryonic blood contains significant numbers of definitive hematopoietic progenitors immediately prior to liver development.

There is a daily logarithmic increase in CFU-GM and BFU-E in the liver between 10 and 13 days, paralleled by an extensive expansion of CFU-S. HSC detected in long-term competitive repopulation assays were reported at E12 in the liver, increasing 38-fold to E16 and decreasing thereafter. Limiting dilution long-term repopulation assays have demonstrated at least one stem cell, as detected by the competitive repopulating in the liver, circulation, AGM region, and YS at E11, with an increase in the liver to a 50-fold increased assay at E12 and a 250-fold by E13 (Figure 23-3). The 24-hour seeding efficiency of murine fetal liver HSC into adult marrow, as measured by limiting dilution competitive repopulation assays, is ~10%, which is essentially identical to the seeding of adult marrow HSC. Thus, ~10 HSC initiate hematopoisis by seeding the hepatic rudiment. These numbers could be explained by colonization from the

AGM, but at E12 the YS makes a significant contribution, and these HSC may not require processing in the AGM, but can mature *in situ*. Relative to adult bone marrow, fetal liver HSC provide long-term lympho-myeloid repopulation of adult mice five-fold more efficiently than adult marrow, a fact explained by their seven-fold higher concentration, and the observation that fetal HSC clones generated ~3-fold more cells than marrow HSC.

In conclusion, the liver appears to be colonized by an early wave of committed and multipotent progenitors and macrophages that may be predominantly of YS origin, and two waves of HSC, the initial one from the YS via the AGM arriving at E10, reaching a maximum at E11, and disappearing by E13, and on E12 a second wave arriving directly from the YS with a contribution from the placenta (Figure 22-5). It is possible that angioblasts also migrate from the yolk sac into the liver to initiate hepatic vasculogenesis. There is no evidence of hemangioblasts or hematogenic endothelium in fetal liver, and sorted hepatic endothelial cells isolated immediately prior to onset of human hepatic hematopoiesis (E27) were devoid of hematopoietic potential.

Ontogeny of Bone Marrow

The primordium of the bone marrow cavity develops following penetration by perichondrial mesenchymal cells and blood vessels into the zone of calcified cartilage in the central region of the long bones. Hypertrophic chondrocytes secrete VEGF that recruits vascular cells to penetrate the perichondrium and bring along osteoblast precursors and circulating hematopoietic cells, including primitive macrophages. Following resorption of the cartilaginous matrix, the developing marrow cavity appears as a network of connective tissue and a plexus of widely-dilated veins. Marrow hematopoiesis is initiated by accumulation of large numbers of undifferentiated basophilic blast cells within the dilate marrow capillaries, beginning at E11–12 in chick, E17 in mouse, and E70–77 in humans. Subsequently, separate and distinct areas of erythropoiesis and granulopoiesis develop. Avian parabiosis studies demonstrated that circulating stem cells colonize the developing marrow beginning at E11–12. Following intravenous injection of tritiated thymidine-labeled embryonic YS or spleen cells into chick embryos, significant numbers of labeled blast cells localized in the marrow at E11. Cells from these tissues also repopulated the marrow when injected into irradiated embryos. In the mouse, CFU-GM/BFU-E and CFU-S and HSC appear in the femoral marrow at E17, and the populations double every 34 hours. The marrow progressively expands in the first two months of postnatal life, associated with a decline in hepatic hematopoiesis in the first week of life and a decline of splenic hematopoiesis after the third week.

Proof that fetal liver stem/progenitor cells were responsible for colonizing the marrow was provided in studies in which rats were injected *in utero* at E16 with a retroviral vector. Clonal identification of viral integration sites showed that fetal liver-derived clones appeared in the marrow and circulated throughout the life of the animals. The role of SDF-1 produced by marrow stromal cells in the chemoattraction of CXCR4+ HSC is well-established in the adult, and the failure of development of marrow hematopoiesis in mice with inactivation of the CXCR4/SDF-1 pathway strongly suggests that the initial wave of HSC migration is also SDF-1 dependent.

Ontogeny of the Spleen

The splenic primordium appears as a dense syncytial-like mesenchymal thickening in the dorsal mesogastrium. The mesenchymal condensation is interspersed with vascular spaces where circulating blood comes into direct contact with mesenchymal reticulum cells. At the earliest stage, corresponding to E8 in chick embryos and E13 in mouse, large immature cells characterized by intense cytoplasmic basophila and prominent nucleoli are observed in both the vascular spaces and the perivascular mesenchyme of the spleen. Within 24 hours, these basophilic cells appear scattered throughout the mesenchyme and frequently extend long tails of cytoplasm between the reticulum cells for some distance from the main body of the cells. By 72 hours, granulopoiesis is extensive, together with erythropoietic foci. Lymphopoiesis is initiated around birth, and myelopoietic activity progressively declines thereafter. Sex chromosome marker studies in parabiosed and twin embryos demonstrated extensive chimerism in the splenic rudiment of the chick embryo as early as E12. Cell-labeling studies confirmed that circulating cells colonized the avian splenic rudiment as early as E8, and together with embryonic hematopoietic cell reconstitution of irradiated embryos, indicated that splenic colonizing cells were present in the YS and circulation at the time of initiation of splenic hematopoiesis. Labeled thymic lymphocytes began to localize in the spleen with high efficiency by E17, coinciding with the initiation of lymphopoiesis.

The origin of cells initially colonizing the mammalian spleen rudiment is most likely the fetal liver, since the AGM and yolk sac are no longer hematopoietic at this stage, and the marrow has not yet developed. CFU-GM/BFU-E, CFU-S, and HSC are detected by E15 in the splenic rudiment and increase in absolute numbers through to three weeks postnatally, then progressively decline as the spleen ceases to be a myelopoietic organ.

CELL MIGRATION TO PRIMARY LYMPHOID ORGANS

Ontogeny of the Thymus

The thymus is an example of interaction between mesenchymal (neural-crest derived) and epithelial (endodermal)

tissues. Notch signaling may play a role in the induction of thymic epithelium. In the mouse embryo at E9.5, Notch receptors and the Notch ligand, Jagged1, are expressed in the third pharyngeal pouch, at the initiation of thymic organogenesis. The thymic anlage at E11 consists of stratified epithelium that, over the following 24 hours, changes to clustered epithelium and begins to express high levels of MHC class II antigen and of Delta1, the Notch ligand that plays a critical role in T-cell development. Comparable development of the thymic rudiment is seen at ~35 days in the human embryo and 7–8 days in the chick embryo. Chromosome marker studies in parabiotic chick embryos demonstrated very high levels of thymic lymphoid chimerism following establishment of a YS vascular union at 6–7 days of incubation, but not following a later established chorioallantoic vascular anastamosis, indicating that circulating stem cells colonized the rudiment at 6–7 days. Subsequent studies showed that the avian thymus is colonized in three waves during embryogenesis at E6, E12, and E18. Progenitors in the first wave came in part from para-aortic foci, with the second and subsequent waves coming from marrow and, to a minor extent, spleen.

The role of the YS as a source of thymic immigrant cells was suggested by studies in which 7-day YS cells were injected into irradiated chick embryos and colonized the thymus. However, sex-mismatched YS and embryo chimeras generated at 33 to 55 hours of incubation showed that the thymus, as well as the bursa, spleen, and marrow, were populated by cells of the sex of embryo and not by YS. Studies in chick-quail chimeras also showed that the primary lymphoid organs were colonized by cells that originate in the para-aortic region. It appears that, in the avian system, the YS is colonized at a very early stage by cells originating in the para-aortic region, and like the mammalian fetal liver, may serve as a site for expansion of lymphoid progenitors that secondarily migrate to the thymus and bursa of Fabricius. During initial thymic colonization (beginning E11 in the mouse, E7 in the chick), which precedes the onset of vascularization by 48 hours, blood-borne precursors must leave adjacent pharyngeal vessels, traverse perithymic mesenchyme and basement membrane surrounding the epithelial rudiment to enter the thymus (Figure 23-4). Approximately 20 T-cell precursors enter the thymus at E11–12, 300 between E12–13, and 3000 between E13–14.

Evidence for thymic chemotactic factors have been provided in functional transfilter cell migration studies that have shown that alymphoid thymic lobes attract cells from fetal liver fragments. MHC class II+ epithelial cells are source of chemoattractant factors, and response is dependent on G-coupled receptors. A number of chemokines and their receptors are expressed either on immigrant cells or epithelium, and one example, TECK, is chemotactic for thymocytes. Chemokine signaling can induce expression of metalloproteinases (MMP9) on immigrant cells that serve to digest extracellular matrix and basement membrane material, and facilitate penetration of the avascular epithelium. The failure of thymic development in the *Nude* mouse is due to a loss of function mutation in the *Foxn1* transcription factor that is essential for thymic epithelial development, and results in failure of lymphoid precursors to enter the epithelium from the perithymic mesenchyme.

The lymphopoietic paths of intrathymic development occur over two weeks with four phenotypically and genetically distinct phases. The first stage (lineage double negative stage 1) is CD4−ve, CD8−ve, CD25−ve, CD44hi and gives rise to T, B, dendritic cell (DC), and NK; the second stage is CD4−ve, CD8−ve, CD25+, CD44hiT, or DC only. Stage 3 is CD4−ve, CD8−ve, CD25+, CD44lo and absolutely committed to T. The final stage is pre-CD4+, CD8+, CD25−. A 4000-fold expansion occurs in the first three phases, and a 250-fold expansion in the early pre-CD4+, CD8+double positive stage.

Origin and Commitment of Thymic Colonizing Cells

The YS and AGM region and the early stage of fetal liver are all sources of lympho-myeloid stem cells (HSC) at the time of thymic colonization. Yet extensive studies using *in vitro* thymic lobe cultures to identify thymic colonizing cells suggest that they are not HSC, and are to some degree T-committed prior to entry into the thymus. Lymphoid commitment may coincide with the first appearance of hematopoietic clusters in the vascular endothelium of the AGM region at E9.5–10. At this stage the CD45+, cKit+, CD34+ population contains multilineage progenitors, as well as progenitors restricted to T (pT) or T/NK plus macrophage (pTm), B (pB), or B plus macrophage (pBm), but not pBT. A common lymphoid progenitor (CLP) identified in adult mouse marrow as a precursor of both B and T lineage can be distinguished from the HSC and common myeloid progenitor (CMP) by expression of the IL7Rα, in addition to being Sca-1+, cKit+, Lin−ve. It appears that, in fetal development, a CLP stage comparable to the adult does not exist, indeed, recent studies have failed to confirm the existence of a CLP even in adult hematopoiesis. The fetal pT cells have T-cell receptor (TCR) rearrangements and appear in the fetal blood and liver at E11–12, where they greatly outnumber pB. This coincides with the period of initial thymic colonization when pT in the liver initially increases and then rapidly declines in number, while pB, initially rare, rapidly increases in number (Figure 23-4).

Cells isolated from the perithymic mesenchyme during initial colonization were IL7Ra+, Lin−ve. Consistent with the paucity of Notch ligand in the fetal liver environment, expression of Notch target genes, *Hes-1* and *pre-Tα*, were not detectable in either fetal liver or perithymic IL7Rα+, Lin−ve populations, indicating that Notch signaling was not activated in these cells prior to thymic entry. In contrast, Notch target genes were detected in precursors that

had entered the epithelial environment where the Notch ligands Jagged and Delta are readily detected. This suggests that a Notch influence on the T-lineage does not occur until cells enter the thymus. However, it is also possible that prethymic commitment to the T-lineage is induced by factors other than Notch, but is only revealed as a result of Notch signaling in the thymus.

To understand lymphoid lineage specification from the HSC, it is best to think of it as a progressive bias along the T, B, or myeloid pathway without abrupt transition steps. The first cell to enter the thymus is predominantly T-directed, but retains some myeloid and B potential. The latter has been revealed by the ability of these cells to undergo delayed B-cell development in culture and by the appearance of B-cell development in the thymus of mice with null mutations of Delta. The pB and the adult CLP are more biased to B- than T-differentiation, and the former retain some myeloid potential. The expression of Pax5 transcription factor has been correlated with B-cell differentiation and progressive down-regulation of myeloid and T-specific genes.

Ontogeny of B Lymphocytes

Avian Bursa of Fabricius

In the avian system, the bursa of Fabricius primordium appears by E5 as an epithelial thickening at the ventrocaudal contact of the cloaca with external ectodermal epithelium. As both bursal and thymic anlagen develop at sites of endodermal–ectodermal interaction, this origin may be of some fundamental importance in the early induction of primary lymphoid organs. By E10–11 longitudinal folds of lining epithelium project into the lumen, and by E12–13 epithelial buds project into the underlying mesenchyme. Bursal lymphopoiesis begins by E14, and at this stage numbers of large basophilic cells are observed in the blood vessels of the mesenchyme adjacent to the epithelial buds. These cells transit through the epithelial basement membrane and localize within the epithelium follicle, where they proliferate extensively, differentiating into typical bursal B lymphocytes. Chromosome marker studies in parabiosed embryos and embryonic grafts of bursal rudiments demonstrated that the bursa was colonized by circulating lymphoid precursors that entered at E13–14. Injection of embryos at E10–14 with tritiated thymidine-labeled embryonic YS, spleen marrow, and blood cells (but not thymocytes) showed rapid localization of labeled basophilic cells in the bursal epithelium, with subsequent proliferation and lymphoid differentiation. The "receptivity" of the bursal epithelium for labeled cells declined abruptly at E15. Parallel injection studies into E14-irradiated embryos using sex chromosome marking confirmed that the above embryonic hematopoietic tissues, but not the thymus, contained bursal lymphoid precursors. By using specific markers expressed on restricted B- and T-precursors, it was shown that cells present on the E14 spleen or marrow that colonized the bursa upon injection were already B-committed with VJ recombination, and distinct from T-lineage precursors. Since such B-restricted precursors appear to be present in the circulation and in all hematopoietic organs at the time of bursal colonization, the precise site of B-lineage commitment is unclear. As a result of sex-mismatched and chick-quail yolk sac-embryo chimeras, it is known that the B-precursors ultimately derive from the intraembryonic para-aortic region, possibly as early as E2, although their subsequent expansion and B-commitment may occur within the YS and then the spleen.

Mammalian B-Cell Ontogeny

The fetal liver has long been considered the initial site of B-cell commitment, beginning at E14 in the mouse, and from then on, the B progenitors expand in a synchronous wave-like pattern reaching a peak in the perinatal stage. It was also generally accepted that early B-cell progenitors expressed CD45R/B220 prior to CD19 expression. However, it has become recognized that B-lineage gene expression (DJH rearrangements and Ig germline transcripts) could be detected in P-Sp/AGM and liver before E12.5. Furthermore, Rag-2, Rag-1, and CD19 gene expression has been found at E9 in YS and P-Sp. By functional and phenotypic analysis, AA4.1+, FcgR+ or AA4.1+, Sca-1+, B220−ve cells with macrophage potential can be detected in E12.5–13 fetal liver. A novel population of cKit+, AA4.1+, CD34+, Sca-1−ve, CD45+, CD19+ cells (70% expressing IL7Ra, but negative for CD45R/B220) were detected in E11 P-Sp/AGM and liver that differentiate exclusively into B cells. Numerically, at E11 these very early pro-B progenitors comprised ~100 in the AGM region and ~500 in the liver, with smaller numbers in the blood and yolk sac (Figure 23-4).

The use of co-culture on marrow stromal lines such as S17 or ST2, often with the addition of cytokines such as IL7 and cKit ligand, have been used to assay for B lymphoid potential. Such studies have identified the emergence of B- or B/macrophage-restricted progenitors in the AGM region at E10, coinciding with the development of the aortic hematopoietic clusters. The number of progenitors capable of generating B-cells in the AGM at E10 (30–35 somite pairs) was 10 and increased 400-fold by E12 where they were located in the fetal liver within the CD45+, Kit+, Sca-1lo, IL7Ra+ population.

There has been a long, and as yet unresolved, debate concerning the role of the YS in generating pB. Early studies with Ig allotype markers showed that YS cells transplanted into adult mice generated B-cells, and *in utero* injection of E8–10 YS resulted in postnatal B- and T-chimerism. Chromosome-marked E11 YS cells were shown to provide long-term lymphoid (T and B) and myeloid

engraftment in mice. Cells from YS as early as E8–E9, when co-cultured on marrow stroma, have been reported to generate cells with B- and T-lymphoid potential as measured *in vitro*, and following transplantation into immune deficient mice. The variable and often conflicting data relating to ontogenetic origin of B-cells can be attributed, in part, to culture variables, the use of different stromal lines, and particularly the use of supplemental cytokines such as IL-7 and c-Kit ligand. Certain of these conditions may provide an environment capable of supporting lymphoid differentiation from hemogenic endothelium or hemangioblasts; others may support differentiation from lymphomyeloid stem cells and pMix; and yet others can expand only those cells that have undergone B-cell commitment (pB or pBm). By restricting the criteria for a committed B-cell progenitor to cells expressing CD45, and probably CD19, IL7Rα, and c-Kit, they first appear within the E10 AGM region where they may traffic to the YS, as well as the early liver. It is unknown whether the more primitive cell populations in the YS that have B-lymphoid potential can migrate into the embryo and undergo pB commitment in the AGM region.

Migration of B progenitors from the AGM to the liver begins at E10, with subsequent increase due to continued immigration and intrahepatic expansion (Figure 22-4). Since HSC are also colonizing the liver at this time, the possibility of intrahepatic generation of additional pB from these more primitive cells cannot be excluded.

KEY WORDS

AGM region Aorto-gonad mesonephros region. The first site of intraembryonic hematopoiesis and the site of origin of hematopoietic stem cells that can function in the adult.

Hemangioblast Common progenitor of both hematopoietic and endothelial lineage. Present in the yolk sac blood islands and in the floor of the dorsal aorta in early development. Presence in the adult marrow is controversial.

Hematopoietic stem cell Self-renewing primitive cell capable of generating all blood cell lineages, including lymphoid and myeloid populations.

Progenitor cells Lineage-restricted primitive hematopoietic and lymphoid precursors, generated from the pluripotent stem cell, but with no self-renewal potential.

Yolk sac blood islands First site of both primitive and definitive hematopoietic development in mammals.

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Chapter 24

Hematopoietic Stem Cells

George Q. Daley

EMBRYONIC STEM CELLS AND EMBRYONIC HEMATOPOIESIS

Over two decades of research in mice has established that embryonic stem cells (ESCs) can give rise to all differentiated cell types in the adult organism, and that ESCs are thus pluripotent. *In vitro*, ESCs undergo spontaneous aggregation and differentiate to form cystic embryoid bodies (EBs). These teratoma-like structures consist of semi-organized tissues, including contractile cardiac myocytes, striated skeletal muscle, neuronal rosettes, and hemoglobin-containing blood islands. In the last decade and a half, this *in vitro* system has been exploited to study differentiation events in a number of tissues, and has begun to be used for discovery and characterization of small molecule pharmaceuticals. The availability of ES cells from the human might make it possible to produce specific differentiated cell types for replacement cell therapies to treat a host of degenerative diseases.

Stem cells from the embryo have fundamentally distinct properties when compared to stem cells from somatic tissues of the developed organism. The hematopoietic stem cell (HSC) is the best-characterized somatic stem cell in the adult. This rare cell, residing in the bone marrow, gives rise to all blood cell lineages and will reconstitute the lympho-hematopoietic system when transplanted into lethally irradiated animals. Bone marrow transplantation is widely employed for the treatment of congenital, malignant, and degenerative diseases. Although adult HSCs are an important target for genetic modification, success has been limited by difficulty expressing genes in HSCs, and by the challenge of maintaining and expanding HSCs in culture. Moreover, recent concerns have been raised about the safety of gene therapy with retroviruses. Thus, harnessing ES cells as a source of HSCs would make it easier to genetically-modify stem cell populations *ex vivo*, to discover small molecules that impact blood development, to study genetic and epigenetic influences on hematopoietic cell fate, and to empower preclinical models for gene and cellular therapy.

A critical question is whether current methods for *in vitro* differentiation of ESCs produce HSCs capable of long-term blood formation in adults. All protocols for *in vitro* differentiation of ES cells published to date appear to recapitulate the yolk sac stage of hematopoietic commitment, with questionable developmental maturation into adult-type somatic HSCs. The first blood cells detected in the yolk sac of the embryo and in EBs *in vitro* are primitive nucleated erythrocytes. These cells express embryonic forms of hemoglobin with a left-shifted oxyhemoglobin dissociation curve, adapted to the low-oxygen environment of the embryo. Embryonic forms of hemoglobin serve as markers of primitive, embryonic erythropoiesis. Later in development, both yolk sac and EBs produce a variety of more differentiated myeloid cell types and enucleated red blood cells that express adult globins and are typical of circulating mature blood. This suggests that yolk sac precursors are capable of making the transition from the primitive to definitive hematopoietic programs, but the extent to which yolk sac-derived progenitors contribute long-term to hematopoiesis in the adult remains controversial. Experimental manipulation of yolk sac blood progenitors can reveal a latent potential for hematopoietic engraftment in adults. When marked yolk sac cells from one embryo are transplanted into other embryos, yolk sac progenitors can contribute to blood formation in the adult. Yet yolk sac progenitors fail to engraft if injected directly into the adult. However, when directly injected into the liver of myeloablated newborn mice, highly purified CD34+/c–Kit+ progenitors isolated from mouse yolk sac will provide long-term blood production throughout adulthood. Apparently, the newborn liver retains the embryonic hematopoietic microenvironment, and supports the developmental maturation of the yolk sac stem cells. Yolk sac cells can also engraft in irradiated mice if they are first cultured on a supportive stromal cell line taken from the aorta–gonad–mesonephros region of the embryo. Here once again, the yolk sac cells can be "educated" by the stromal cells to adopt an adult profile. So indeed, hematopoietic progenitors from yolk sac appear capable of sustaining

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hematopoiesis in adults, but based on recent evidence from a number of groups, it appears likely that yolk sac progenitors contribute to embryonic blood formation, and thereafter yield to a distinct source of definitive HSCs that arise within the embryo proper. Compelling evidence suggests that the definitive HSCs responsible for lifelong hematopoiesis arise chiefly from a separate and distinct source in the aorta–gonad–mesonephros region of the developing embryo.

BLOOD FORMATION IN EMBRYOID BODIES

As for yolk sac progenitors, it has proven exceedingly difficult to demonstrate that ES-derived hematopoietic progenitors can repopulate adult mice. This resistance of embryonic progenitors from EBs to engraft in the adult is reminiscent of the block to engraftment observed for yolk sac progenitors, and likewise is believed to reflect the developmental immaturity of ES-derived HSCs, and the different microenvironments of the embryo and the adult. Whether *in vitro* differentiation of ESCs will promote formation of AGM-like HSCs remains unresolved. In order to model hematopoietic transplantation from ES cell sources, this challenge must be overcome.

Although EBs show a temporal wave of primitive, followed by definitive, hematopoiesis, the nature of the ES-derived HSCs has been a subject of great interest. The delineation of hematopoietic development within EBs has been pioneered by Keller and colleagues, whose seminal contributions have made the hematopoietic program among the most well-defined aspects of *in vitro* ES cell differentiation. Their work has defined the most primitive hematopoietic progenitor in EBs as the blast colony forming cell (BL-CFC), a transient cell with both primitive erythroid potential and the capacity to generate definitive erythrocytes and multilineage myeloid colonies upon replating. It also has the potential to form endothelial cells and, as such, has been defined as the hemangioblast. To date there is no data demonstrating lymphoid potential for this cell type. Only one group has reported repopulation of irradiated adult mice with cells taken from EBs fortuitously timed to harbor the maximal number of BL-CFCs, but in this report there were no markers to demonstrate that lymphoid and myeloid cells developed from a single cell committed to the hematopoietic lineage. Thus, to date the relationship of the BL-CFC to definitive HSCs remains ill-defined, and whether EBs support the development of AGM-like HSCs remains an open question.

TRANSFORMATION OF AN EB-DERIVED HSC BY BCR/ABL

We set out to address the question of whether a lymphoid–myeloid HSC developed within differentiating EBs by attempting to transform that putative cell. We borrowed from our experience with the disease chronic myeloid leukemia (CML), the classical pathologic condition of adult hematopoietic stem cells that is caused by the BCR/ABL oncoprotein. Several biological properties unique to the BCR/ABL oncoprotein made it particularly well-suited for addressing the nature of primitive blood progenitors within EBs. Induction of CML requires expression in pluripotent hematopoietic stem cells. Multi-lineage hematopoiesis is monoclonal in most patients at the time of CML diagnosis, demonstrating that the fusion oncoprotein BCR/ABL endows leukemic stem cells with a competitive repopulation advantage over normal stem cells. Despite expression in a wide array of tissues, transgenic mice that carry BCR/ABL in their germline develop only hematopoietic malignancies, reflecting the tropism of BCR/ABL for hematopoietic cell types and the sparing of nonhematopoietic tissues. Patients with CML harbor the Philadelphia chromosome translocation in both lymphoid and myeloid lineages, showing that the multi-lineage differentiation of stem cells is preserved despite BCR/ABL transformation.

We hypothesized that BCR/ABL would enable us to transform an ES-derived HSC, engraft mice, and determine the extent of lymphoid and myeloid differentiation *in vivo*. We introduced BCR/ABL into differentiated murine ES cells and cultured a primitive hematopoietic blast cell that generated nucleated erythroblasts *in vitro*, mimicking yolk sac blood formation. We picked and expanded single-cell clones of these cells, verified clonality by retroviral integration, injected irradiated mice, and observed successful lymphoid–myeloid engraftment in primary and secondary animals. The erythroid progenitors from engrafted mice expressed only adult globins, suggesting that the cells underwent developmental maturation to the definitive hematopoietic program *in vivo*. BCR/ABL expression enabled adult engraftment by altering the cell's homing properties, complementing a missing cytokine signal, or blocking apoptosis, allowing the ES-derivatives to acclimatize to the adult microenvironment and to differentiate into multiple hematopoietic lineages. These results provided the first definitive demonstration of the embryonic HSC (e-HSC) that arises *in vitro* during ES differentiation. This cell is a common progenitor of both primitive embryonic erythropoiesis (yolk sac type) and the definitive adult lymphoid–myeloid hematopoietic stem cell. Although the BCR/ABL-transformed clones produce colonies with a comparable morphology to the BL-CFC and show primitive erythroid potential, their precise relationship to the hemangioblast is unclear, as the BCR/ABL-transformed clones do not appear to have endothelial potential.

PROMOTING HEMATOPOIETIC ENGRAFTMENT WITH STAT5 AND HOXB4

Although BCR/ABL transformation targets a rare cell in ES cell cultures with lymphoid–myeloid developmental potential, the engrafted mice succumbed to leukemia, prompting us to

explore means for isolating the e-HSC without inducing the transformed phenotype. Our approach to generating normal, nontransformed blood progenitors from ES cells involved a combination of two strategies: (1) expression of single proteins in signaling pathways activated by BCR/ABL (e.g., STAT5), postulating that activation of downstream targets would be less disruptive to cell physiology than transformation by the complete oncoprotein; and (2) conditional expression of candidate genes using a novel ES cell line engineered to express the gene of interest from a tetracycline regulated promoter such that genetic effects could be induced and then reversed. The Ainv15 ESC line we created expresses the tet-dependent transcriptional transactivator protein from an active genomic locus (*ROSA26*).

Any gene of interest can be inserted with high targeting efficiency into an expression cassette located within the active *HPRT* gene locus. Genes that are targeted correctly become resistant to neomycin (G418), and the inserted gene is expressed only in the presence of the potent tetracycline analog doxycycline, and can be rapidly silenced following doxycycline removal. We chose to express the *STAT5* transcriptional regulator and the homeobox gene *HoxB4* in this system, owing to the central role of STAT5 in BCR/ABL and cytokine receptor signaling, and to extensive prior evidence from the Humphries group of the role of *Hox* genes in hematopoiesis and the unique properties of *HoxB4*, which was previously shown to enhance hematopoietic engraftment without inducing leukemia. We differentiated the modified ES cells into EBs and activated gene expression by adding doxycycline to the culture medium between differentiation days four and six, timed to coincide with the maximal generation of primitive multipotential hematopoietic colonies. After six days, the EBs were dissociated, and the cells were cultured on the OP9 stromal cell line, previously shown to enhance production of hematopoietic progenitors from mES cells. Expansion of hematopoietic blast cells was observed upon *STAT5* and *HoxB4* gene induction, and vigorously growing colonies of hematopoietic cells were detected only in the presence of doxycycline. These cells were harvested, plated in semisolid media plus cytokines, and shown to produce a variety of blood cell colony types, with only the most primitive multipotential colonies significantly expanded. The cells, which also express the green fluorescent protein (GFP), were injected intravenously into irradiated syngeneic or immunodeficient mice.

Contributions of GFP+ cells to the peripheral blood were then monitored by flow cytometry, and specific lymphoid and myeloid cell populations were scored by antibody staining against specific cell surface differentiation antigens, by forward and side scatter properties, and by direct microscopic examination of cells cyto-centrifuged onto cover slips. In these experiments, both *STAT5* and *HoxB4*-expressing cells engrafted in mice and generated both lymphoid and myeloid populations in circulating blood. Interestingly, contributions of the *STAT5*-stimulated cells appeared to be transient, despite continued gene induction *in vivo* (through inclusion of doxycycline in the drinking water of the mice). Engraftment with *HoxB4*-expressing cells persisted in primary animals, even in the absence of gene induction, and the cells of primary animals could be transplanted into secondary animals, suggesting self-renewal of a long-term reconstituting hematopoietic stem cell. Examination of peripheral blood smears from engrafted mice showed no evidence of abnormal hematopoiesis, although rare animals succumbed to hematologic malignancies from donor cells, suggesting some tendency for the genetically-modified ESCs to undergo transformation *in vivo*. Retroviral delivery of *HoxB4* directly to populations of cells dissociated from EBs after four to six days of differentiation also succeeded in generating expanding cultures of hematopoietic cells that engrafted in irradiated mice. These data demonstrate that expression of the *STAT5* or *HoxB4* gene in differentiating cultures of ES cells yields hematopoietic engraftment in irradiated mice, with *HoxB4* showing the most promise for stable engraftment in primary and secondary animals.

The mechanisms by which *STAT5*, and particularly *HoxB4*, drive hematopoietic engraftment from ESCs remain unclear. By driving cell proliferation, both genes might serve to increase the numbers of otherwise vanishingly rare HSCs above a threshold of detection. Alternatively, *HoxB4* might be altering cell fate, a known effect of homeobox genes, by promoting a transition from primitive to definitive HSC fate. RT-PCR analysis of *HoxB4*-expressing EB-derived cells, in comparison to hematopoietic progenitors isolated from precirculation yolk sac, confirmed the detection of markers of definitive hematopoiesis in *HoxB4* expressing cells, including adult-type globin. Moreover, *HoxB4*-transduced cells expressed *CXCR4*, the chemokine receptor implicated in hematopoietic stem cell homing to the bone marrow, and *Tel*, the transcription factor implicated in migration from the fetal liver to the adult bone marrow microenvironment. *HoxB4* does not appear to be expressed in the precirculation yolk sac, but is detected in primitive populations of CD34+ bone marrow cells from the adult. We therefore also tested whether expression of the *HoxB4* gene by retroviral infection in yolk sac progenitors would endow them with engraftment potential in adults. As for EB-derived cells, *HoxB4* expression in yolk sac progenitors induced dramatic expansion on OP9 stromal cultures and stable hematopoietic engraftment in adult mice. Yolk sac-derived progenitors could also be transplanted into secondary animals. These data support the hypothesis that activation of *HoxB4* endows embryonic hematopoietic progenitors with the potential to engraft in adult hematopoietic microenvironments, and therefore may be critical to the transition from the embryonic to adult hematopoietic program.

Lymphoid potential and transient lymphoid reconstitution of engrafted immunodeficient mice has been demonstrated, showing convincingly that EB-derived cells can

show full hematopoietic differentiation potential. Native EB-cell derived hematopoietic progenitors function at best inefficiently to reconstitute the adult host, or may develop in only limited numbers under idiosyncratic culture conditions. If indeed distinct progenitors contribute to primitive yolk sac and definitive AGM-type hematopoiesis in the embryo, then distinct progenitors might arise at spatially and temporally distinct sites during EB formation from ES cells *in vitro*. Identifying the precise culture conditions that promote differentiation of ESCs into robust adult-type definitive HSCs *in vitro* remains a critical goal.

To date, gene modification has been required to demonstrate development of lymphoid–myeloid HSCs from differentiating ES cells *in vitro*, yet even gene modification results in inefficient engraftment. Given the technical challenges and risks inherent in genetic modification, it would be preferable to derive engraftable HSCs in as natural a process as possible, by mimicking the developmental pathways of the embryo. Applying principles that specify blood formation to *in vitro* systems might enable enhanced blood formation in a safe and efficient manner.

PROMOTING BLOOD FORMATION *IN VITRO* WITH EMBRYONIC MORPHOGENS

During gastrulation in the mouse, secreted signaling molecules induce distinct cell fates from developing mesoderm. The earliest stages of blood formation occur in the extraembryonic mesoderm of the yolk sac, where blood islands form surrounded by endothelial cells and closely opposed to the visceral (primitive) endoderm. Several of the players that regulate the transcriptional control of hematopoiesis have been identified (e.g., *SCL*, *AML1/CBFa2*, and several *Hox* genes), largely due to their involvement at translocation breakpoints in leukemia. Gene knockout studies of these factors have validated their role in hematopoiesis. More recently, a small number of secreted factors have been identified that act as early embryonic inducers of mesodermal fate in the hematopoietic lineage. The most interesting of these are hedgehog factors and bone morphogenetic protein 4 (BMP4).

Indian hedgehog (Ihh) is a member of the hedgehog family of signaling molecules that play diverse roles in patterning early embryonic events. Baron and colleagues have shown that early hematopoietic activity in the developing murine yolk sac is dependent on signals from the adjacent primitive/visceral endoderm. Recently, they demonstrated that Ihh was produced by visceral endoderm sufficient to mediate this induction. Ihh could respecify neuroectoderm to hematopoietic fate, and blocking Ihh function by anti-hh antibodies abrogated hematopoietic development. Baron and colleagues showed that Ihh induction of hematopoiesis in murine embryo explants led to expression of BMP4. BMP4, a member of the TGF-β family, has been implicated as a potent ventralizing factor and inducer of hematopoietic mesoderm in Xenopus development. A related hedgehog factor, Sonic hedgehog (Shh), has been shown by Bhatia and colleagues to enhance the expansion of human hematopoietic repopulating cells assayed by transplantation in immunodeficient mice. BMP4 and other members of the BMP family have also been implicated in regulation of proliferation and survival of primitive human hematopoietic populations. Antibodies to hh and noggin, a specific antagonist of BMP4 signaling, abrogated the proliferative effects of Shh in human hematopoietic cell culture. It has been shown that Ihh is expressed by visceral endoderm in developing EBs. There is a single report that addition of BMP4 to differentiating cultures of rhesus ES cells augments formation of hematopoietic clusters. Most recently, Bhatia and colleagues have shown that BMP4 can enhance hematopoietic potential from human ES cells.

These studies offer the first hints that directed differentiation of human ES cells into HSCs might be feasible. However, numerous questions persist: will HSCs derived from hES cells function normally? Will they reconstitute normal immune function and remain nontumorigenic *in vivo*? Can immunologic issues be circumvented through nuclear replacement or gene modification? The hurdles for therapeutic applications of ES-derived cells remain high, but no matter what, the *in vitro* differentiation system will remain an important model for investigations of blood formation and embryonic development.

KEY WORDS

Embryoid bodies Cystic teratoma-like structures consisting of semi-organized tissues representing all three embryonic germ layers. Formed when embryonic stem cells are removed from culture conditions that inhibit differentiation, and are allowed to aggregate and differentiate.

Hemangioblast (blast colony forming cell- BL-CFC) A long-theorized, but only recently identified, embryonic cell representing a common progenitor of both the endothelial and hematopoietic lineages. Can be readily detected in differentiating cultures of embryonic stem cells as the first mesodermal element committed to the hematopoietic lineage. Assayed by colony formation in methycellulose supplemented with vascular endothelial growth factor and stem cell factor (the BL-CFC).

Primitive versus definitive hematopoiesis The first wave of embryonic blood development. It occurs in the yolk sac and consists principally of nucleated erythrocytes that express embryonic globins. The primitive wave is believed to supply the needs of the embryo and to be later supplanted by a definitive class of hematopoietic stem cells that arise in the para-aortic region of the developing embryo proper. Definitive hematopoiesis generates mature myeloid and lymphoid lineages for the life of the animal.

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Chapter 25

Red Blood Cells

Shi-Jiang Lu, Jennifer S. Park, Qiang Feng, and Robert Lanza

ERYTHROPOIESIS

Erythropoiesis is a complex multi-step process that involves the differentiation of hematopoietic stem cells (HSCs) into mature erythrocytes (red blood cells, RBCs). Erythropoiesis begins when a multi-potential HSC undergoes erythroid unilineage commitment and continues through the proliferation and terminal maturation of the erythroid committed progenitor cell. This process is highly regulated and involves multiple steps, including the differentiation of early erythroid progenitors (burst-forming units-erythroid, BFU-E) to late erythroid progenitors (colony-forming units-erythroid, CFU-E), and finally leads to the terminal enucleation and maturation of RBCs. Because of its functional importance in early development, erythroid lineage appears first during ontogeny in the blood island of the yolk sac. Later, different anatomic sites, such as the fetal liver and finally the bone marrow (BM), are recruited for erythropoiesis.

During mammalian embryonic development, erythropoiesis progresses through distinct stages. The hematopoietic progenitors appear in four waves. The first wave is the formation of blood islands in the extraembryonic mesoderm (yolk sac). These primitive erythroblasts are large, nucleated and express embryonic hemoglobin type $\varepsilon_2\zeta_2$. The second wave occurs in the intraembryonic mesoderm, specifically in the region of the para-aorta splanchpleural (P-Sp), which later becomes the aorta-gonad mesonephros (AGM). The third wave occurs in the fetal liver, and finally the long-lasting hematopoietic activity homes to the BM. The erythrocytes from the yolk sac enter the circulation mostly as nucleated cells, whereas the cells entering the circulation from fetal liver and BM are enucleated.

Definitive erythropoiesis in the fetal liver features the production of enucleated RBCs that quickly become dominant in embryonic circulation. The switch of hemoglobin to fetal types ($\alpha_2\gamma_2$) also occurs at the initiation of definitive erythropoiesis. However, recent reports show that yolk sac-derived primitive erythroblasts can also enucleate in the circulation of a mouse embryo and persist throughout gestation. In adults, all blood cell types, including lymphocytes, myeloid cells, and RBCs are derived from HSCs residing in bone marrow. Within the bone marrow, erythroid progenitors undergo a series of maturation stages prior to becoming reticulocytes. Each stage is associated with the defining morphological characteristics in the cytoplasm and the nucleus of the precursor cells distinguishable after Wright's stain. In the final stage of the maturation process, along with a dramatic reduction in cell size, basophilic pronormoblasts lose their nuclei to become reticulocytes, which then enter the blood circulation as fully mature RBCs.

The *in vivo* development of the mammalian blood system has been well-studied, including numerous research advances involving mouse embryonic stem cells (mES) and subsequent gene knockout mice models. Inactivation/deletion of genes required for the formation of the circulation system has contributed to a better understanding of the biology of blood formation from the early embryonic stage to adulthood. The complex process of erythropoiesis proceeds with dramatic changes in gene expression and cell morphology. Erythropoietin (EPO) has been identified as a key factor in a feedback loop mechanism. EPO is a hormone produced in the kidney and liver that stimulates the production of RBCs in the bone marrow in response to hypoxia stress. One mechanism of EPO's action is to promote the survival and expansion of erythroid progenitors. Other factors, such as retinoic acid and dexamethasone, are also shown to be important during erythropoiesis. In the murine system, the transcription factor GATA1 has been shown to be an essential regulator of erythroid lineage commitment, as well as terminal differentiation.

ERYTHROCYTES GENERATED FROM ADULT STEM CELLS *IN VITRO*

The need for transfusable RBCs has spurred research into generating erythrocytes *in vitro* from a variety of adult stem cell sources, including cord blood (CB), peripheral blood (PB) and bone marrow. CD34+ cells from cord blood, peripheral blood, and bone marrow have been isolated and

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differentiated into erythrocytes with 95% purity after culture using EPO, stem cell factor (SCF), and interleukin-3 (IL-3). Co-culturing with a mouse MS-5 stromal cell line or human MSCs results in enucleation, which is a hallmark of mature RBCs. These erythrocytes have similar properties to normal RBCs, including membrane deformation capacity, enzymatic content, and balanced adult/fetal hemoglobin, with the ability to bind and release oxygen. The erythrocytes survive *in vivo* in NOD-SCID mice and are detected after three days. Stem cells from peripheral blood and bone marrow have limited expansion capacity (29000 and 16500-fold, respectively) compared to those from cord blood (140000-fold). However, the advantages of using cells from adult peripheral blood and bone marrow are that they may be easier to obtain, and display mature forms of hemoglobin, whereas those from cord blood only express fetal globins.

ERYTHROCYTES GENERATED FROM MOUSE EMBRYONIC STEM CELLS

The *in vitro* differentiation and expansion of erythrocytes from mES cells has also been achieved. With no additional exogenous cytokines, co-culture of mES cells with OP9, a mouse stromal cell line deficient in macrophage colony stimulating factor (M-CSF), is sufficient to induce erythroid cell production. The presence of M-CSF has inhibitory effects on the differentiation of mES cells to blood cells other than macrophages. Erythropoiesis of mES cultured with OP9 comes in two waves, the first wave includes large nucleated cells, deemed to be primitive erythrocytes, and the second wave includes either small nucleated erythroblasts or enucleated mature blood cells morphologically identical to definitive erythrocytes, containing only adult globins.

An embryoid body (EB)-based culture system can result in as much as five million-fold expansion of mES cell-generated erythroblasts within 10 weeks. EBs are formed after plating mES cells in ultra-low binding plates, in which mES cells are unable to attach to the surface and instead cluster and attach to each other in 3D suspension, mimicking the *in vivo* environment. EBs are cultured in the presence of serum, dissociated and purified in serum-free liquid culture, and terminally differentiated using erythroid cytokines. Erythrocytes generated using this method express adult hemoglobin and are enucleated when exposed to a high concentration of Epo, transferrin and insulin. When these erythroblasts are transplanted into mice, they show faster maturation and superior morphology of enucleated cells compared to those cultured under *in vitro* conditions. Erythroblasts also mature significantly faster in fetal liver than in *in vitro* culture conditions, suggesting that although full maturation can be achieved *in vitro*, a better understanding of the erythroid differentiation mechanisms, as well as the development of improved expansion protocols, will be required to further advance this field.

ERYTHROCYTES GENERATED FROM HUMAN EMBRYONIC STEM CELLS

Human ES cells (hESC) offer the possibility of creating an inexhaustible source of universal (O^-) blood. However, since one unit or pint of blood contains approximately two trillion cells, the ability to produce large-scale RBCs in an efficient and cost-effective manner will be critical for clinical application. Another significant challenge will be to achieve full maturation *in vitro*. Compared to studies using mES cells, erythrocytes generated from hESCs are mostly nucleated, expressing little or no adult β-globin. However, recently, there have been significant advances in the large-scale differentiation of hESCs towards RBCs.

In vitro differentiation of erythroblasts has been achieved by co-culturing hES cells with stromal lines, followed by isolation of CD34+ cells and further expansion/differentiation. This approach in general generates erythroblasts on a small scale. A method using serum and human fetal liver cell co-culture followed by mouse MS-5 co-culture can generate a relatively large amount of mostly nucleated and large erythroblasts. No β-globin chain expression is observed, suggesting the cells are primitive/fetal erythroblasts. From 50000 CD34+ cells, 5000- to 10000-fold amplification is obtained; however, it is unclear how many hESCs are needed to generate 50000 CD34+ cells. The use of serum and two different feeder cells makes this a non-ideal procedure for scale-up. Although serum can provide growth factors needed for RBC differentiation, its undefined nature can introduce difficulties in terms of reproducibility and low efficiency.

We have reported the generation of functional hemangioblasts from hESCs using a serum-free culture system at relatively large-scale and high purity (>95%). The hemangioblast is the common precursor cell to hematopoietic and endothelial cell lineages. One of the characteristics of this unique progenitor cell is its highly efficient generation of BFU-E and CFU-E colonies when cultured in serum-free colony-forming cell medium. This prompted us to investigate whether hemangioblasts can be used as an intermediate to generate erythrocytes on a clinically relevant scale.

There are three critical elements to achieve high efficiency of RBC generation: (1) efficient generation of hemangioblasts without disruption of their colony forming environment; (2) expansion of hemangioblasts to erythroblasts in a high cell density; and (3) culture in semi-solid media containing methylcellulose to provide optimal conditions for maximum expansion capacity and high erythroid purity. Using this approach, we have generated approximately 10^{10} to 10^{11} erythroid cells per six-well plate of hESCs (Figure 25-1). This erythroid population is mostly nucleated, nearly 100% positive for glycophorin A (CD235a), expresses ABO antigen, and has a very high content of hemoglobin (Figure 25-2). A low percentage (<5%) of spontaneous enucleated cells show identical size and morphology with mature human RBCs. These

hESC-derived erythroid cells behave similarly to normal RBCs, with comparable oxygen equilibrium curves, and response to changes in pH and 2,3-diphosphoglycerate. Although the cells mainly express fetal and embryonic globin chains (Figure 25-1E), they also possess the capacity to express the adult definitive β-globin chain on further maturation *in vitro*. PCR and globin chain-specific immunofluorescent analysis show that the cells increased expression of β-globin from 0% to 15% after *in vitro* culture (Figure 25-2). A separate study recently demonstrated that almost all the erythrocytes derived from hESCs co-cultured with mouse fetal liver stromal cells express the adult β-globin chain after extended culture *in vitro*. Importantly, when the hemangioblasts used in our studies are purified from semi-solid media, transferred to erythroid

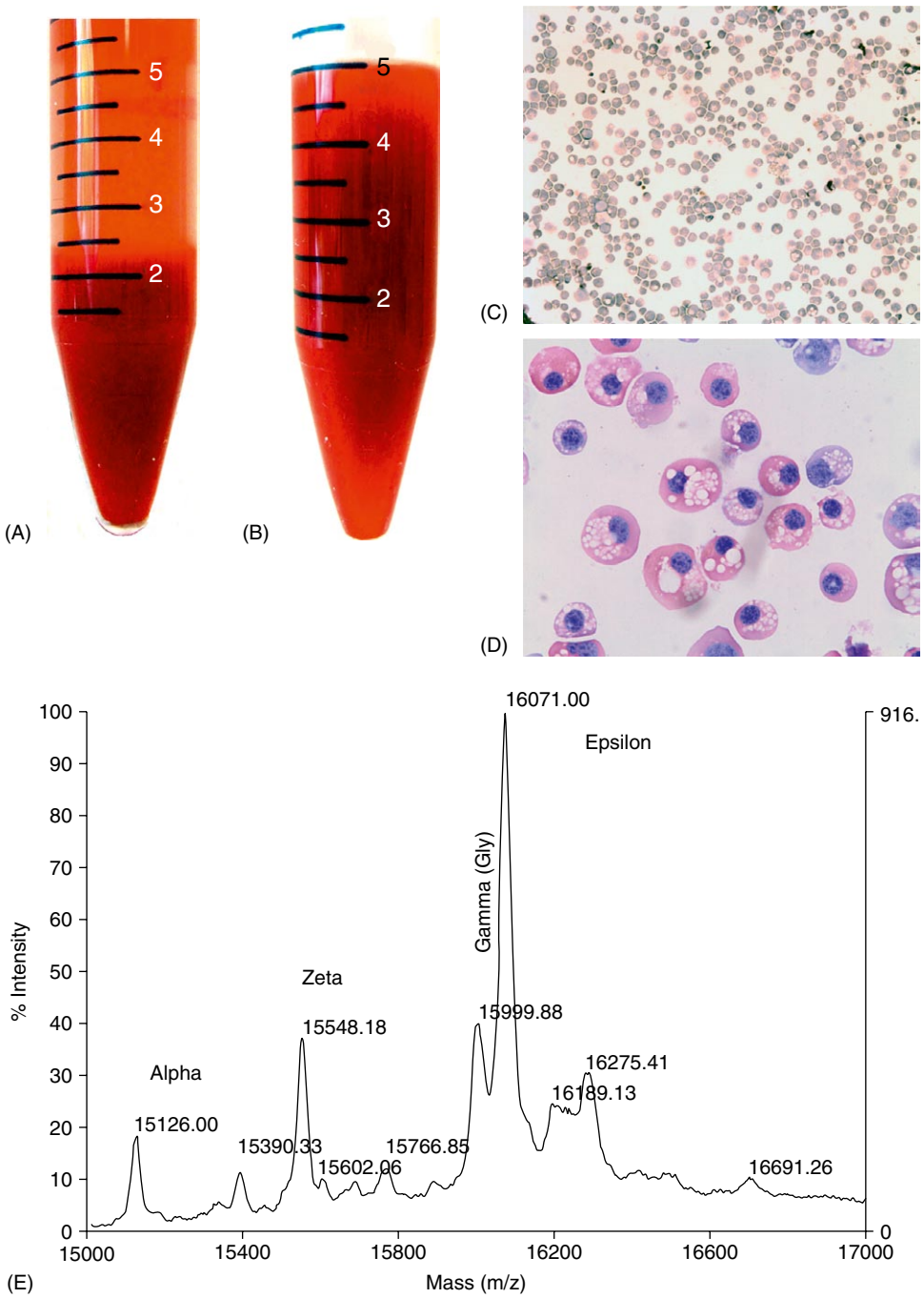


FIGURE 25-1 Large-scale production of erythroid cells from hESCs. (A) Erythroid cells (pellet) derived from 2×10^6 human ESCs; (B) Erythroid cells from panel A were resuspended in equivalent hematocrit of human whole blood; (C) (200 ×) and (D) (1000 ×), Morphology of erythroid cells derived from human ESCs. (E) Electrospray ionization mass spectra of globin chains in hemoglobins from hESC-derived erythroid cells, confirming the presence of α, ζ, ε, and $^{G}\gamma$ globins. The observed molecular weight for each of the globins is shown.

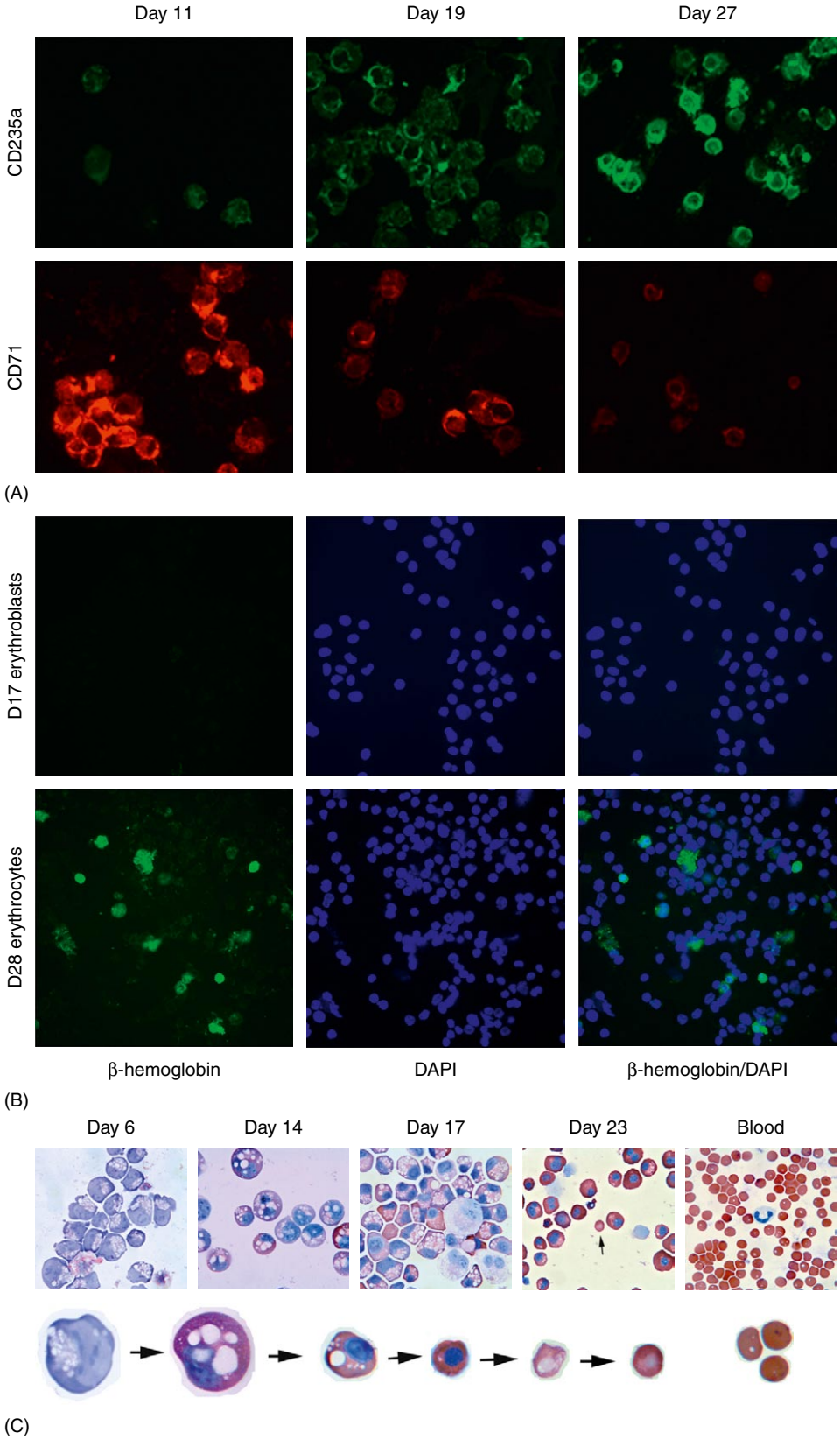


FIGURE 25-2 Maturation of hESC-derived erythroid cells mimic erythroid development. (A) Expression of CD235a, a mature erythrocyte marker, increases with time; and CD71, an immature red blood cell marker, shows a decrease in expression over time (200 ×). (B) Expression of β-globin chain in hESC-derived erythroid cells. Cytospin samples of hESC-derived erythroid cells collected from day 17 and day 28 differentiation and maturation cultures were stained with human β-globin chain specific antibody (200 ×). (C) Progressive maturation of hESC-derived erythroid cells *in vitro*. Progressive morphological changes from blast cells to erythroblasts, and eventually matured erythrocytes, is accompanied by significant increase of hemoglobin and decrease in size during their *in vitro* differentiation and maturation. Cells were stained with both Wright-Giemsa and benzidine.

liquid culture, and co-cultured with OP9 stromal cells or MSCs, the erythroblasts undergo multiple maturation events, including a progressive decrease in size, increase in glycophorin A expression, and chromatin and nuclear condensation. This process results in extrusion of the pycnotic nuclei in up to 65% of the cells generating RBCs with a diameter of approximately 6–8 μm (Figures 25-2 and 25-3). These data suggest that hESC-derived erythroblasts could be a promising source of functional RBCs in the future.

ENUCLEATION OF ERYTHROID CELLS

Maturation of erythroblasts is indicated by loss of transferrin receptor CD71, expression of CD235a, and finally enucleation. It has been extensively shown that co-culture with stromal cells promotes *in vitro* hematopoietic cell growth and expansion. In bone marrow, where adult hematopoiesis occurs, MSCs are an important compartment of the hematopoietic environment, secreting soluble factors that signal development of hematopoietic progenitors. MS-5 has been shown to be important in supporting hematopoietic development, excreting IL-6 and other important soluble factors. Not only are the secretion of soluble factors from stromal cells important in signaling to hematopoietic cells, but cell–cell contact has been shown to be even more important than soluble factors alone in generating large numbers of hematopoietic cells. Even more importantly, stromal contact has been shown to be crucial in promoting enucleation in CD34+ cord blood, peripheral blood, bone marrow-derived RBCs with up to 100% enucleation. Cells in stromal-conditioned media without physical cell–cell contacts do not enucleate. The stroma could be responsible for engulfing nuclei and other organelles present in the immature erythroblasts. Since it has been shown that stromal-conditioned media is not sufficient to progress RBC maturation, direct cell–cell contacts may provide the necessary cues for RBC maturation in erythropoietic development. Alternatively, enucleated erythroblasts from cord blood stem cells have been produced at a rate of 77% without using stromal cells. VEGF and IGF-II may have contributed to priming the cells to extrude their nucleus, as well as enucleation medium containing Plasmanate, D-mannitol, adenine, disodium hydrogenphosphate dodecahydrate, and mifepristone.

The molecular and cellular mechanisms involved in the erythroid differentiation that lead to the terminal enucleation and maturation of RBCs are poorly defined, even though enucleation of erythroblasts was structurally studied by electron microscopy almost half a century ago. It has been suggested that enucleation is the result of asymmetric cell division, involving extrusion of a pycnotic nucleus enveloped by the plasma membrane, and that Rac GTPases

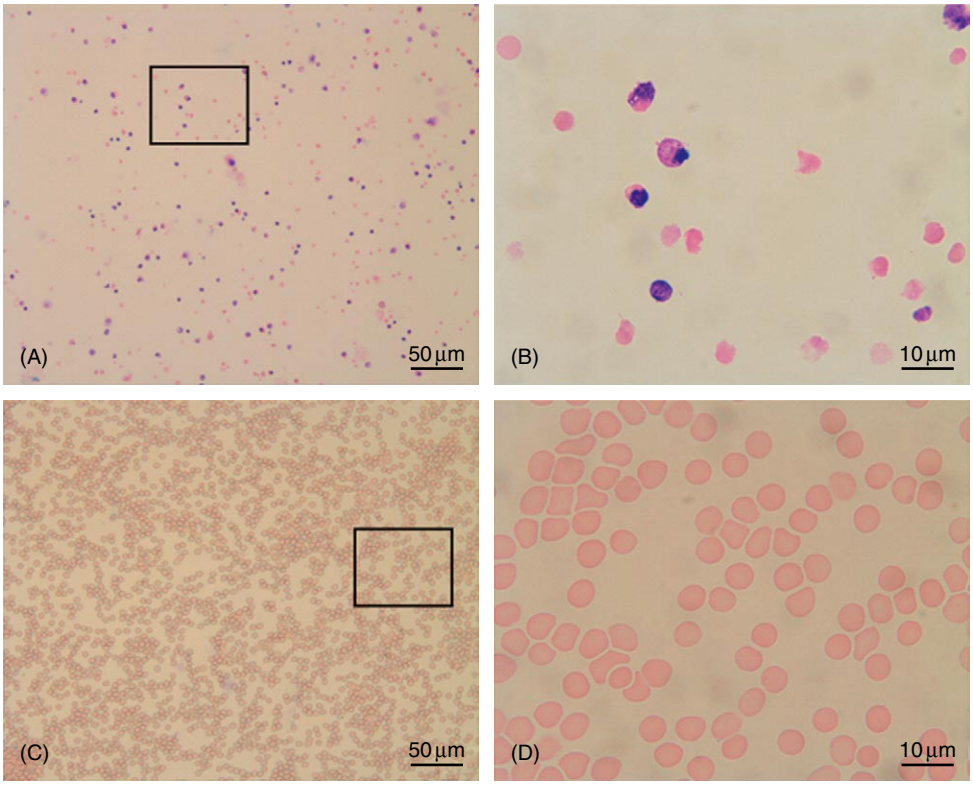


FIGURE 25-3 Enucleation of hESC-derived erythroid cells *in vitro*. Erythroid cells derived from human ESCs (A, B) were cultured *in vitro* for three weeks with stromal cells in Stemline II media with supplements. Cells were cytospun and stained with Wright-Giemsa dye, along with red blood cells from human blood (C, D); B, D are high magnification areas from the rectangles in A, C, respectively. Scale bar = 10 μm.

and their effector mDia2 play important roles in the process. Direct contact of erythroblasts with macrophages promotes nuclear extrusion, and knockout of erythroblast-macrophage-protein (Emp) results in the failure of enucleation. Studies in a mouse genetic model indicate that tumor suppressor gene Rb can promote definitive erythropoiesis through the regulation of fetal liver marcophages. However, although macrophages play a role in the maturation of erythroblasts, macrophages have recently been shown to be neither sufficient nor required for RBC enucleation, at least in the mouse system. More mechanistic enucleation studies need to be performed in order to be able to achieve complete maturation of stem cell-derived RBCs for clinical applications.

SUMMARY

Limitations in the supply of RBCs can have potentially life-threatening consequences for patients with massive blood loss due to trauma or surgery, or who suffer from diseases that cause severe anemia. Although alternative sources of progenitors for the generation of large-scale transfusable RBCs have been investigated, including cord blood, bone marrow and peripheral blood, it is clear that even after expansion and differentiation, these progenitors represent donor-limited sources of RBCs. Moreover, the low prevalence of O^--type blood in the general population ($<8\%$ in Western countries and $<0.3\%$ in Asian countries) further intensifies the consequences of blood shortages for emergency situations where blood typing may not be possible. hESCs and human induced pluripotent stem (iPS) cells represent a new source of stem cells that can be propagated and expanded *in vitro* indefinitely, providing a potentially inexhaustible and donorless source of RBCs for human therapy.

KEY WORDS

BFU-E Burst forming unit-erythroid, the earliest known erythroid precursor cell that eventually differentiates into erythrocytes. Produces a colony containing greater than 200 erythroblasts in a hematopoietic colony assay.

CFU-E Colony forming unit-erythroid, a late erythroid precursor cell (after BFU-E) that eventually differentiates into erythrocytes. Produces one to two clusters containing a total of 8–200 erythroblasts in a hematopoietic colony assay.

Embryoid bodies (EBs) Cystic aggregates of ES cells when plated in ultra-low surface culture plastics and allowed to differentiate. Consists of semi-organized tissues representing all three germ layers.

Hemangioblast A common progenitor for both hematopoietic and vascular lineages. Can be easily detected in differentiating cultures of both mouse and human ES cells.

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Chapter 26

Cell Differentiation in the Skeleton

Gerard Karsenty

INTRODUCTION

The vertebrate skeleton is composed of two specialized tissues, cartilage and bone, surrounding and protecting a third one, the bone marrow. Each of these two skeletal tissues contains a specific cell type of mesenchymal origin, chondrocytes in cartilage and bone-forming osteoblasts in bone. Both chondrocytes and osteoblasts fulfill unique functions that are critical for the growth, maintenance, and integrity of the skeleton. Chondrocytes are required for longitudinal growth of the bones, and synthesize the cartilaginous scaffolds on which osteoblasts can deposit bone matrix both during development and postnatal growth. Osteoblasts synthesize a collagen-rich matrix that has the unique property to become eventually mineralized. Defects of differentiation or function of any of these two cell types have tremendous consequences, as reflected by the large number of genetic and acquired diseases of the skeleton. Several transcription factors, as well as a complex interplay of secreted molecules, have been identified that control the progression from a pluripotent progenitor to a functional cell.

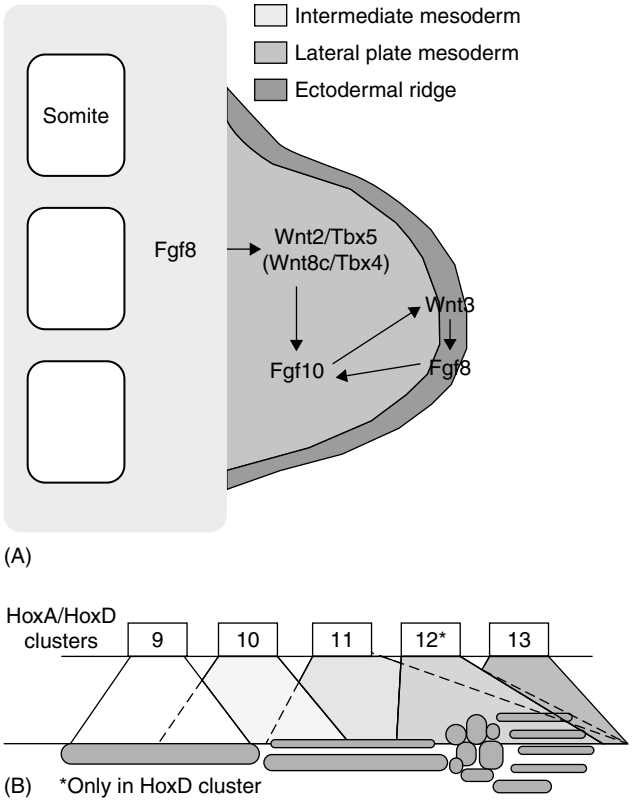


FIGURE 26-1 Patterning of skeletal elements in the limbs. (A) An initial series of interactions between Fgf and Wnt signaling pathways induce limb bud formation and early outgrowth. (B) Limb skeletal elements are later specified by the proximo-distal expression of distinct genes of the HoxA and HoxD clusters. The area of influence of each particular set of genes (boxes) is marked with a distinct shade of grey.

SKELETOGENESIS

Formation of the vertebrate skeleton begins with aggregation of undifferentiated mesenchymal cells in structures that prefigure each future skeletal element. These skeletal condensations, also called anlage, form around 9.5 days postcoitum (dpc) during mouse development, and become visible histologically around 10.5dpc. Assembly of these skeletal condensations is orchestrated by molecules that control migration and association of undifferentiated cells (Figure 26-1). Although some differences exist between craniofacial, axial, and limb patterning, the pathways controlling this patterning process show many similarities. Once formed, skeletal condensations can form bones, following either an intramembranous or an endochondral ossification process. Bones formed by intramembranous ossification include the frontal, parietal, and parts of the temporal and occipital bones, the majority of the facial bones, and the lateral part of the clavicles. In these flat

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bones cells of the condensations expand to form a membranous structure; then cells differentiate directly into osteoblasts. They maintain and then increase their secretion of type I collagen while initiating expression of noncollagenous proteins more specific of the osteoblast such as Bone sialo protein and Osteocalcin.

In the rest of the skeleton, skeletogenesis proceeds by endochondral ossification, a multi-step process. By 12.5dpc of mouse development, cells in the center of the skeletal condensations differentiate into chondrocytes, expressing type II collagen and aggrecan. Between 14.5dpc and 16.5dpc, as the condensation elongates, the most inner chondrocytes differentiate into hypertrophic chondrocytes expressing type X collagen. In the meantime, cells at the periphery of the condensation flatten, and begin to differentiate into osteoblasts that express type I collagen. This structure, termed the bone collar, is most apparent in the region closer from the hypertrophic zone of the condensation. The second step of the endochondral ossification process is marked by two coordinated events. While hypertrophic chondrocytes start to die through apoptosis, blood vessels invade the region they occupied, bringing in differentiating osteoblasts from the bone collar. The bone matrix synthesized by osteoblasts then replaces the cartilaginous matrix. This ossification spreads centripetally until much of the cartilage is replaced. Only a small region at either end of the growing bone will remain cartilaginous. This structure, called the growth plate cartilage, will control longitudinal growth of the bone until the end of puberty. As the newly-deposited bone matrix mineralizes, osteoclast precursors coming from the bloodstream invade the center of the forming structure, and begin to differentiate under the influence of factors secreted by osteoblast progenitors. They start to resorb the bone matrix, forming the internal space that will contain the bone marrow.

CHONDROCYTE DIFFERENTIATION

The study of chondrogenesis has largely benefited from two particularities of this process. First, differentiation of chondrocytes induces significant morphological changes that facilitate identification of distinct maturational stages. Second, most of the differentiation process is constantly recapitulated in the growth plate cartilage in skeletal element developing by endochondral ossification (Figure 26-2). Cells at the earliest stage of maturation are small, and they are located in the most distal area, termed the reserve or resting zone. They are considered to be chondrocyte progenitors. Immediately below this resting zone a proliferative zone emerges, where cells are slightly larger, flatter, and rapidly dividing. The length of these columns of rapidly dividing chondrocytes is a major determinant of longitudinal bone growth. Proliferating chondrocytes express type II collagen and aggrecan. However, the most

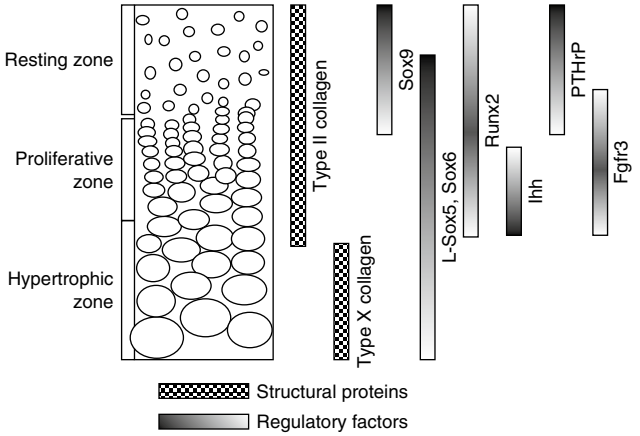


FIGURE 26-2 Cell distribution and gene expression at the embryonic growth plate cartilage.

proximal cells (also called prehypertrophic chondrocytes) also express Indian hedgehog (Ihh), and the receptor for both parathyroid hormone (PTH), and PTH-related peptide (PTHrP). The most proximal zone appearing on the growth plate cartilage contains hypertrophic chondrocytes. They express type X collagen, but not type II collagen, and eventually they undergo apoptosis.

Sox9, a member of the high-mobility-group (HMG) family of transcription factors, is the earliest known molecule to be required for chondrocyte specification. Sox9 was first identified as the gene inactivated in campomelic dysplasia (CD), a dominant genetic disorder characterized by skeletal malformation and sex reversal. Subsequently, Sox9 was found to activate the expression of the type II, type IX, and type XI collagen genes in chondrocytes (61–64). Mouse genetic studies clarified the role of Sox9 during chondrogenesis. They showed that Sox9−/− cells, but not wild-type (wt) embryonic stem (ES) cells, are excluded from the skeletal condensations, indicating that Sox9 is required for their formation. Moreover, teratomas derived from Sox9−/−, but not from wt ES, failed to develop cartilage in mouse chimeras, and Sox9−/− cells are abnormally sensitive to apoptotic signals. Two other HMG family members, L-Sox5 and Sox6, also play a key role during chondrocyte maturation. Both genes were found to be co-expressed with Sox9 in precartilaginous condensations, but continue to be expressed in hypertrophic chondrocytes. Sox5 and Sox6 are redundant *in vivo*, and single null-mutant mice are virtually normal. However, targeted deletion of both L-Sox5 and Sox6 leads to late embryonic lethality, due to generalized chondrodysplasia, demonstrating their essential role in control of chondrocyte differentiation.

In contrast to this group of activators, NFATl, a member of the family of nuclear factor of activated T-cells, acts as a repressor of chondrocyte differentiation. Indeed, its overexpression in chondrocytic cell lines suppresses expression of chondrocyte molecular markers, and NFAT1-deficient mice

develop ectopic cartilage in the joints. The newly-formed cartilage contains ordered and columnar chondrocytes with distinct morphologies, and is eventually replaced by bone, recapitulating the process of endochondral ossification. FGFs, a class of secreted factors, also inhibit chondrocyte differentiation. Because of the large number of FGF members expressed at every step of skeleton formation, it is via the functional analysis of their receptors (FGFRs) that the role of these factors has been best characterized. Fgfr3 inactivation in mice causes increased chondrocyte proliferation, while activating mutations in FGFRs are found in chondrodystrophic patients with shortened proliferating zone at the growth plate cartilage. These observations indicate that one or several FGFs, acting through this receptor, inhibit chondrocyte proliferation.

Chondrocyte proliferation hypertrophy, the last stage of chondrocyte maturation, is controlled by a negative feedback loop involving two growth factors, PTHrP and Indian hedgehog (Ihh). PTHrP, secreted by the chondrocytes of the perichondrium, signals to the PTHrP receptor (PPR) expressed by proliferating chondrocytes to stimulate their proliferation. When escaping PTHrP spatial range of activity, cells become prehypertrophic; they begin synthesizing Ihh, stop proliferating, and start differentiating in hypertrophic chondrocytes. Accordingly, chondrocyte-specific constitutive expression of PPR in mice causes a delay in conversion of proliferative chondrocyte into hypertrophic chondrocyte, and an activating mutation in the human PPR causes a short-limb dwarfism inherited disorder. In turn, Ihh secretion by prehypertrophic chondrocytes directs cells of perichondrium to up-regulate their synthesis of PTHrP, thereby indirectly slowing down the pace of chondrocyte hypertrophy. The presence of an increased fraction of hypertrophic chondrocytes in Ihh-deficient mice confirmed this role.

One transcription factor, Runx2, formerly Cbfal, also controls hypertrophic differentiation. Runx2 was originally identified for its role as an osteoblast differentiation factor (see below). Besides an absence of osteoblasts, Runx2-deficient mice have proximal long bones populated mostly by resting and proliferative chondrocytes, but they show no hypertrophic chondrocytes, and no type X collagen expression. In contrast, targeted overexpression of Runx2 into nonhypertrophic chondrocytes of wild-type mice, or of Runx2-deficient mice, leads to ectopic endochondral bone formation and specific rescue of the lack of hypertrophic chondrocytes, respectively. This latter rescue occurs without rescue of the arrest of osteoblast differentiation, indicating that Runx2 control of chondrocyte hypertrophy is independent of its osteoblast differentiation ability.

Lastly, it has been shown that hypertrophic chondrocytes control vascular invasion, a step required for continuation of the endochondral ossification process. Indeed, cartilage resorption by MMP9 releases VEGF, an angiogenic factor secreted by hypertrophic chondrocytes, which in turn regulates vascular invasion. Recently, the analysis of mice deficient for connective tissue growth factor (CTGF) showed that this factor would be regulating extracellular matrix remodeling and vasculogenesis, via its control of VEGF and MMP9 expression.

OSTEOBLAST DIFFERENTIATION

Unlike the case for chondrocytes, osteoblast differentiation is not marked by phenotypic changes *in vivo*. The only mark of differentiation is slow to appear and lies outside the cell: fully differentiated osteoblasts can produce a matrix that becomes mineralized. This absence of morphological characteristics at the cellular level implies that one has to rely on gene expression studies to assess osteoblast differentiation. Here again, the osteoblast has only a few specific markers. In fact, there is only one truly osteoblast-specific structural gene, *Osteocalcin*, which is only expressed by fully-differentiated osteoblasts.

Runx2 is the earliest regulator of osteoblast differentiation identified to date. It belongs to a family of transcription factors related to runt, a regulator of neurogenesis and sexual differentiation in *Drosophila*. Runx2 is expressed in every osteoblast progenitor cell, regardless of its embryonic origin. Runx2-deficient mice have a normally patterned skeleton that is entirely cartilaginous. The only skeletal elements missing are those formed through intramembranous ossification. At these sites only a membranous structure exists; since there is no cartilaginous stage in the development of these structures, no cartilaginous scaffold can exist. Interestingly, Runx2 heterozygous mutant mice present hypoplastic clavicles and a severe delay of the closure of the fontanelles. These features recapitulate a human condition called Cleidocranial dysplasia (CCD), and mutations into the *Runx2* gene cause CCD. That Runx2 haploinsufficiency results in a significant phenotype further emphasizes its importance during osteoblast differentiation. Recently, mice harboring an inactivation of Cbfb (a small protein without DNA binding and transcriptional abilities known to interact with Runxl, another Runx factor required for hematopoiesis, lethal only perinatally), were analyzed. In contrast with the identical phenotypical abnormalities observed in Runxl-deficient and classic Cbfb-deficient mice, these mice did not reproduce the skeletal phenotype of the Runx2-deficient mice. In fact, their phenotype was rather similar to Runx2 haploinsufficiency. This observation, and the absence of obvious heterodimerization between Runx2 and Cbfb *in vitro*, suggests that Cbfb may play an accessory role during osteoblast differentiation.

Osx, a zinc finger-containing nuclear factor, has recently been identified as another regulator of osteoblast differentiation. *Osx* is specifically expressed in osteoblasts, and Osx-deficient mice lack osteoblasts. *Osx* is not expressed in Runx2-null mice, yet Runx2 is expressed

normally in Osx-deficient mice, indicating that Osx acts downstream of Runx2. Besides Cbfal and Osx, two broadly expressed members of the API family of transcription factors, DFosB and Fra-1, have been reported to act as positive regulators for osteoblast differentiation. DFosB is an alternative spliced transcript of FosB lacking the FosB C-terminal domain. Generalized overexpression of DFosB in transgenic mice leads to an increase in osteoblast differentiation. Similarly, forced ubiquitous expression of Fra-1 causes an increase in osteoblast proliferation.

Secreted molecules have also been shown to control osteoblast differentiation. For instance, in the absence of Ihh, Runx2 expression is not induced in cells of the bone collar, and osteoblast diffentiation is blocked in skeletal elements undergoing endochondral ossification. These observations indicate that Ihh is a determinant of early osteoblast differentiation, and that this secreted factor may act via its regulation of Runx2 expression. FGFs also regulate osteoblast differentiation. Indeed, inactivation of FGF18 in mice causes a general delay of bone formation, affecting both skeletal elements forming by intramembranous and endochondral ossification.

KEY WORDS

Chondrocyte Cell type of mesenchymal origin present in cartilage; can be nonhypertrophic or hypertrophic.

Mesenchymal condensation Aggregation of undifferentiated mesenchymal cells prefiguring a future skeletal element.

Osteoblasts Cell type of mesenchymal origin present in osteoblast and responsible for bone formation.

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Chapter 27

Human Vascular Progenitor Cells

Ayelet Dar, Sharon Gerecht-Nir, and Joseph Itskovitz-Eldor

HUMAN VASCULAR DEVELOPMENT, MAINTENANCE AND RENEWAL

During the third week of human embryonic development, blood vessels are formed in conjunction with blood islands within the yolk sac mesoderm. During this process, blood islands develop alongside the endoderm, which segregate into individual hemangioblasts that are surrounded by flattened endothelial precursor cells. The hemangioblasts mature into the first blood cells, while the endothelial precursors develop into blood vessel endothelium. At the end of the third week, the entire yolk sac, the chorionic villi, and the connecting stalk are vascularized. New vascular formation, termed vasculogenesis, takes place within the embryo on day 18. During this process, the underlying endoderm secretes substances that cause some cells of the splanchnopleuric mesoderm to differentiate into angioblasts. These mesodermal angioblasts then flatten into endothelial cells and coalesce, resulting in small vesicular structures referred to as angiocysts. The latter fuse to form networks of angioblastic cords that later unite, grow, and invade embryonic tissues to create the arterial, venous, and lymphatic channels. The spread of vessel networks occurs by three processes:

1. Continuous fusion of the angiocysts—vasculogenesis.
2. Sprouting of new vessels from existing ones—angiogenesis.
3. Assimilation of new mesodermal cells into the existing vessel's wall.

Although the yolk sac is the first supplier of blood cells to the embryonic circulation, the role of blood cell production is taken over by a series of embryonic organs, such as the liver, spleen, thymus, and bone marrow. Therefore, two major concepts were suggested: the bipotential hemangioblast produces the primitive erythroid and endothelial progenitor cells, and the hemogenic endothelium gives rise to hematopoietic stem cells and endothelial progenitors. The ability of these progenitors of hematopoiesis (blood cell repopulation), vasculogenesis or angiogenesis will be discussed.

Vasculogenic Embryonic Cells

Hemangioblasts/angiohematopoietic Cells: Endothelial-hematopoietic Common Progenitor Cells

In human embryos, homogenic endothelium can be observed and isolated from both extra-embryonic and intra-embryonic regions. In the human embryo (between the 27th and 40th day of development), a closely packed population of CD34+ cells is found adhered to the ventral side of the aortic endothelium. These cells are characterized by expression of both mature hematopoietic and endothelial cell markers, including CD45, CD34, and CD31, and by absence of GATA-2, GATA-3, c-kit, and flk-1/KDR. In a more recent work, endothelial cells of three- to six-week-old human embryos were found to express CD31, CD34, and vascular endothelial cadherin (VE-Cadherin). CD31+ CD34+ CD45− cells isolated from both the yolk sac and aorta were shown to differentiate into the myelo-lymphoid lineage in culture. In addition, isolated CD34+ CD31− cells from 11- to 12-week-old human embryo differentiated into both CD34+ CD31− and CD34+ CD31+ cells. The latter were found to be capable of forming a network of capillary-like structures.

Recently, a novel cell surface antigen, BB9 (also expressed by stro-1+ bone marrow stromal cells), was shown to identify the earliest angio-hematopoietic precursors in the human embryo. During human embryonic development, BB9 is expressed in all blood-forming tissues, including the yolk sac (day 19 of gestation), para-aortic splanchnopleura (in CD34− CD45− until 26 days of gestation), the aortic progenitors (day 27 of gestation), fetal liver, and in the bone marrow at later developmental

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stages. The dynamic expression of BB9 implies the migration of hemangioblast precursors from the para-aortic splanchnopleura toward the ventral aorta to further differentiate into BB9+ CD34+ CD45+ hematopoietic progenitor cells, or BB9+ CD34+ CD45− endothelial cells. Transplantation assays conducted in an immunodeficient murine model strongly support this notion: only isolated human embryonic BB9+, but not BB9−, could reconstitute hematopoiesis and rescue lethally irradiated mice. In addition, the *in vitro* model of differentiating human embryonic stem (hES) cells was used as a tool for studying human embryogenesis and developmental vasculogenesis. In agreement with the physiological and *in vivo* data, embryoid body differentiation mimics the physiological mesodermal-hemangioblast pathway. In the course of this process, the development of BB9+ ACE+ (angiotensin-converting enzyme) cells toward either hematopoietic or endothelial lineage is regulated by the renin-angiotensin system. In another hES model, bone morphogenetic protein-4 (BMP-4) induced the efficient development of KDR/VEGFR2+ progenitor cells, with both hematopoietic, as well as vascular, potential within 72–96 hours of differentiation. Two distinguished sub-populations emerged from the KDR+ hemangioblasts: one gave rise to primitive erythroid cells, macrophages, and endothelial cells, while the other also produced endothelial cells, but only primitive erythroid ones.

Cardiovascular Progenitor Cells: Cardiac-endothelial-smooth Muscle Cell Common Progenitors

The heart tissue is composed of mesodermal-derived lineages including cardiomyocytes, endothelial, and smooth muscle cells (SMCs). Evidence for a common origin, and the signaling pathways which direct the differentiation of these cell types in early development, are currently available from hES cell models, as well as studies in adult tissues. The earliest evidence in humans was recently shown on umbilical vein endothelium-derived cells that differentiated into SMCs through *in vitro* culturing with a fibroblast growth factor (FGF). Similar to murine embryos, wherein early periendothelial SMCs were observed in association with embryonic endothelial tubes originating from a common progenitor, the usage of an hES cell model revealed even more primitive multipotential cardiovascular progenitor cells. These cells can be directed toward maturation of either cardiac, endothelial, or SMCs in a multi-step exchange of growth factors throughout the culture period: stimulation of Flk-1+ cells with BMP-4, followed by a combination of BMP-4, bFGF and activin-A, resulted in development of multipotent progenitors that could be directed toward either cardiac, endothelial, or SMCs fate by additional defined stimulations.

A more restricted type of progenitor, capable of differentiation towards endothelial and mural (smooth muscle actin-expressing cells) is the VEGFR2+TRA1−ve-Cadherin+ sorted from hES cells that were allowed to differentiate on murine bone marrow stromal cell line (OP9). Following a culture period in the presence of VEGF, re-sorted VEGFR2+ TRA1−ve-Cadherin− cells gained mural cell phenotype in the presence of PDGF-BB. Co-transplantation of both cell types augmented neovascularization in hind limb ischemic mice model.

The identification and induced generation of defined multipotent progenitors in hES cell systems is beneficial for various fields, such as studies of cardiovascular development and signaling pathways that regulate progenitor cells' commitment and maturation. In addition to the ability to obtain virtually unlimited numbers of hES cell-derived cells, these cells can potentially be used to improve and to establish new clinical protocols.

Vascular Progenitors in Adult Tissues

Various studies have been conducted in an attempt to identify an adult hemangioblast, the progenitor for both endothelial and hematopoietic cells. By this methodology, endothelial progenitor cells were isolated from fat tissue, lymphoid organs such as bone marrow, and spleen, as well as CD133+, CD34+, or CD31+ circulating cells from cord blood and peripheral blood of adult human. Upon the onset of endothelial-induced differentiation by various endothelial growth factors (e.g., VEGF, FGF-2, EGF, and ECGF), these populations were capable of endothelial outgrowth with high proliferative capability and endothelial angiogenic functionality in animal models. In the course of endothelial induced differentiation, these cells express endothelial specific markers, such as VE-cadherin or E-selectin. A specific fraction of those cells also expressed Flk-1, which indicated the existence of a population of functional endothelial precursors. A thorough examination revealed that most circulating endothelial cells isolated by their P1H12 expression (endothelial cell surface glycoprotein), originated from vessel walls and have a limited growth capability. In addition, CD34+ KDR+ cells isolated from adult bone marrow were shown to have bi-lineage differentiation potential: they generated hematopoietic cells, endothelial cells, and cells expressing both types of markers. Furthermore, endothelial precursors were also identified in adult human heart tissue. A subset of multipotent cardiovascular c-kit+ cells devoid of hematopoietic lineage markers (CD45, CD34, CD133) differentiated predominantly into cardiac cells and, to a lesser extent, to endothelial and SMCs.

Less is known about the origin and differentiation process of SMCs in adulthood. It was only recently found that SMCs emerge *in vitro* and *in vivo* from CD146+ isolated pericytes-like mesenchymal stem cells. These cells comprise the abluminal endothelial layer of veins or arteries

in multiple adult and fetal human organs (skeletal muscle, pancreas, bone marrow, and placenta) and express SMCs/mesenchymal lineage markers such as CD90, CD105, CD73, and CD44.

HUMAN EMBRYONIC STEM CELLS AS A SOURCE FOR VASCULAR PROGENITORS

Spontaneous Differentiation

Both spontaneous and induced differentiation of hESCs can occur in two-dimensional (2D) and three-dimensional (3D) cultures; i.e., by cell adherence (cell–matrix interactions) and cell–cell contact (cellular communication).

Human embryoid bodies (hEBs) are formed by the spontaneous differentiation of hESCs, and comprise multilineage tissues from endodermal, ectodermal, and mesodermal origin. Several experimental procedures have been developed to explore the endothelial potential of hESCs.

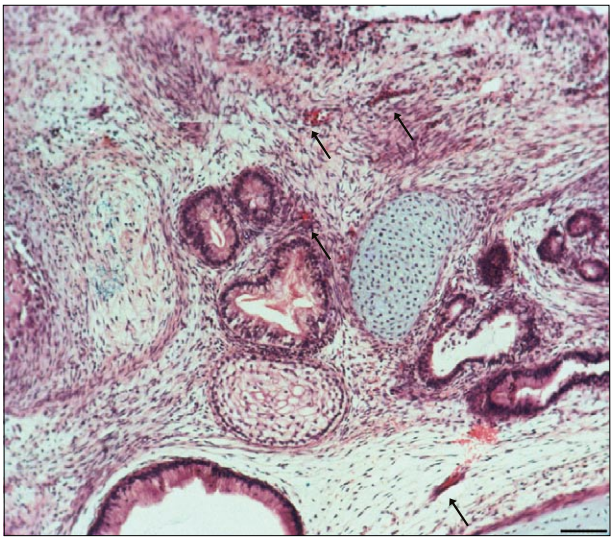
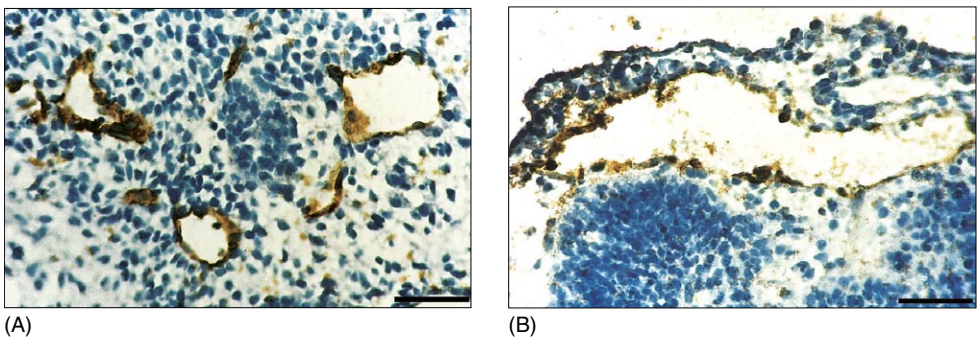


FIGURE 27-1 Teratoma vasculature. Human embryonic stem cells (hESCs) formed teratomas once injected into severe combined immunodeficient (SCID) mice. Various blood vessels (arrows) could be observed within the formed teratoma, including human and mouse-originating vessels. Bar = 100 mm.

Undifferentiated human embryonic stem cells (hESCs) form teratomas once injected into severe combined immunodeficient (SCID) mice. Within these teratomas various blood vessels are formed (Figure 27-1). The small-diameter vessels located at the center of the teratomas originate in humans. Therefore, during teratoma formation from hESCs, two parallel vascular processes occur: (1) angiogenesis of host vasculature into the forming human teratoma; and (2) vasculogenesis of spontaneously differentiating hESCs.

Endothelial cell surface markers, mainly CD34, CD31 (PECAM-1) or VE-cadherin, have been reported to identify subsets of primary endothelium, and have been used in attempts to isolate endothelial immature and maturing cells, based on the kinetics of their expression by EBs in the course of differentiation. CD34, which is considered to be an early human endothelial marker, is clearly expressed by surrounding endothelial cells that form voids within one-month-old hEBs (Figure 27-2). Exploring the early organization of CD34+ cells within 10- to 15-day-old hEBs reveals two types of cell arrangements: the first is a typical three-dimensional (3D) vessel formation, and the second is a cryptic arrangement that is occasionally somewhat difficult to associate with typical 3D-vessel formation (Figure 27-3A). However, both elongated and round cells stained for CD34+ represent both endothelial and hematopoietic cells, respectively (Figure 27-3B). In another model, CD31, which is highly expressed by immature and mature endothelial cells, appears from day four of EB differentiation, and peaks at days 13–15, together with the appearance of vascular networks in hEBs. Sorted out CD34 or CD31 cells are capable of endothelial growth with typical rearrangement in 2D and 3D cultures and vasculogenesis *in vivo*. Exploring the early organization of CD31+ cells within 10- to 15-day-old hEBs revealed a typical 3D-vessel formation in different locations within the hEBs (Figure 27-4A, B). At this time point a few round cells, probably early hematopoietic progenitors (Figure 27-4C), also expressed CD31. However, these markers are not exclusive to the endothelial lineage on the onset of hESC differentiation and/or EB maturation (e.g., CD34 also identifies hematopoietic progenitors), implying the need to develop more complex isolation systems based on



(A) (B)

FIGURE 27-2 Blood vessels in human embryoid bodies (hEBs). Histological sections of one-month-old hEBs stained with anti-CD34 revealed (A) the formation of relatively small vessels and (B) larger blood vessels. Bar = 100 mm.

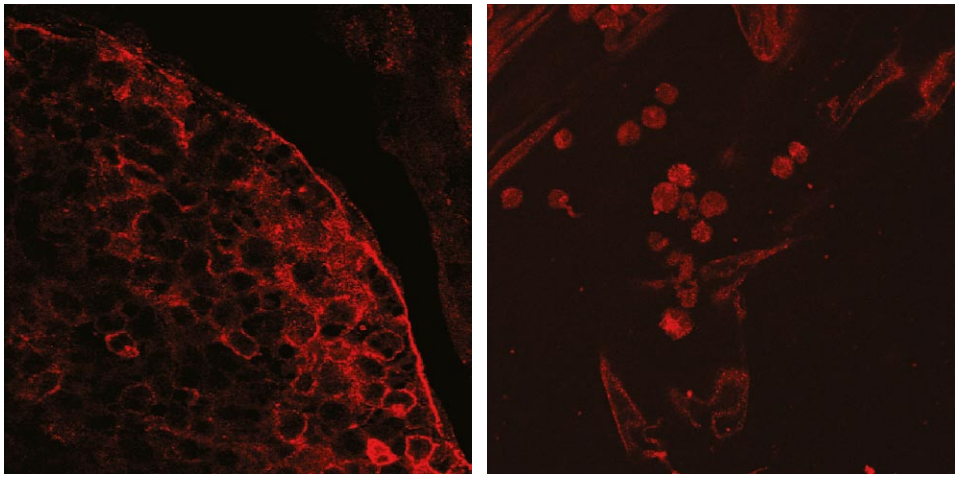


FIGURE 27-3 CD34+ cells in whole-mount human embryoid bodies (hEBs). 10- to 15-day-old EBs stained for CD34 revealed (A) occasionally cryptic, atypical vessel arrangement and (B) positive elongated and round cells.

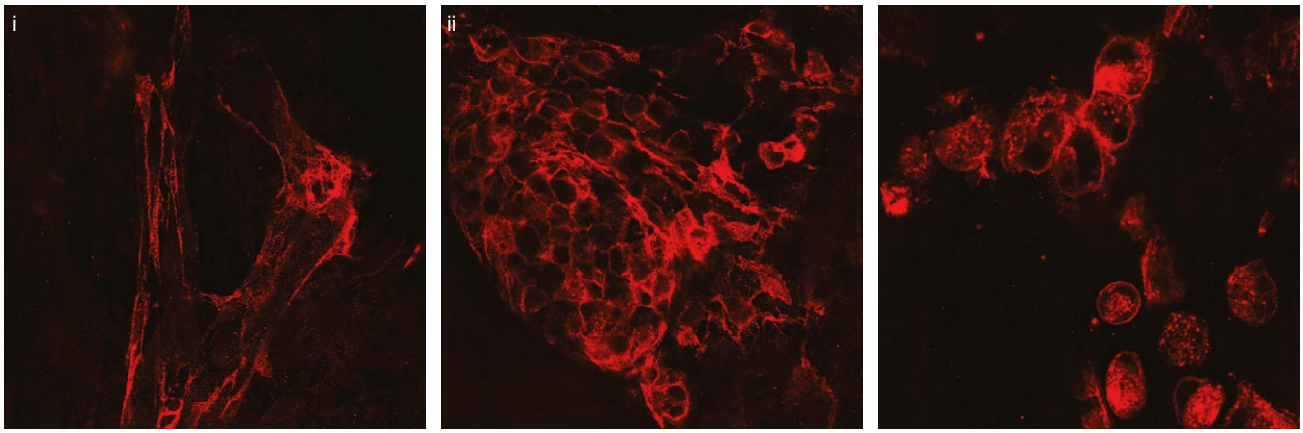


FIGURE 27-4 CD31+ cells in whole-mount human embryoid bodies (hEBs). 10- to 15-day-old-hEBs stained for CD31 revealed (A) different vascular organization within the EBs such as (i) elongated, (ii) curved; and (B) a few positive round cells.

combinations of several markers and/or levels of expression. Thus, in further studies of human vasculogenesis, various vascular markers should be examined and evaluated in the course of spontaneous differentiation.

Induced Differentiation

Approaches for the promotion of specific vascular differentiation of hESCs include the following, and are based on one or a combination of a few endothelial derivation procedures:

1. Genetic manipulation, also known as gene targeting—knockin and knockout specific angiogenic receptors and relevant transcription factors.
2. Exogenic factors—administrating the cell cultures with specific and known angiogenic and hematopoietic factors.
3. Matrix-based cultures—culturing the differentiating cells on matrices known to support vascular cell cultures.
4. Co-culture—culturing the differentiating cells with specific stromal cell lines that may promote vascular differentiation.

Induction of vascular differentiation from hESCs can be achieved by seeding hESCs on semisolid methylcellulose media supplemented with ascorbic acid and ITS, or alternatively on type IV collagen matrices for 14 days with differentiation medium. Collagen type IV culture conditions induced the expression of all vascular endothelial growth factor (VEGF) isomers, VEGF receptors KDR, growth factor Ang2 and its receptor Tie2, and CD31-specific marker of endothelial cells. Some of the differentiated cells formed muscle–vascular arrangements, which were found to express smooth muscle a-actin (SMA). For the induction of a more defined population, single-cell

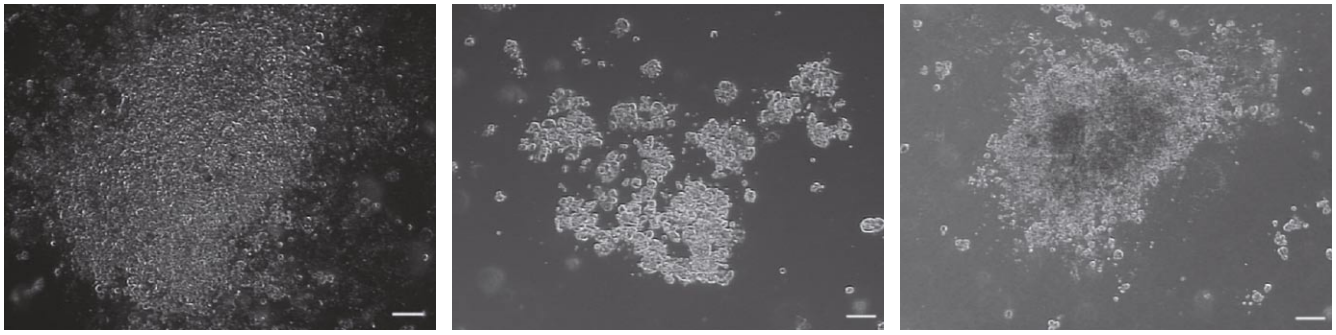


FIGURE 27-5 Hematopoietic colonies. Enriched progenitor population cultivated in semi-solid media formed different types of hematopoietic colonies.

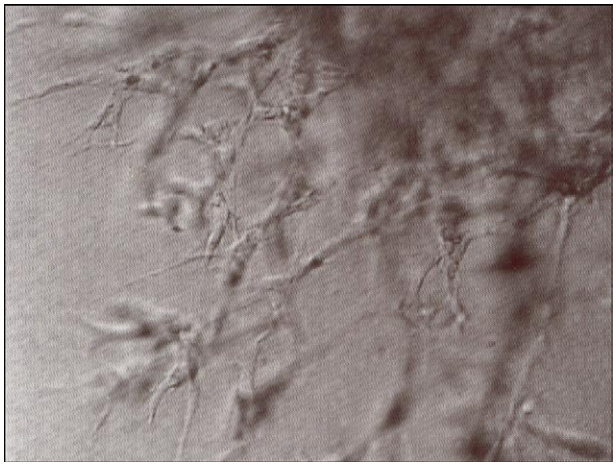


FIGURE 27-6 Tube-like structures and sprouting. An enriched progenitor population seeded within Matrigel sprouted and formed vessel-like structures.

suspensions of hESCs were used. Examination of the differentiated population revealed that the majority of the surviving cells expressed specific endothelial and endothelial-hematopoietic markers. To induce lineage-specific differentiation, administration with human VEGF, platelet-derived growth factor BB (PDGF-BB), or specific hematopoietic cytokines was examined. VEGF treatment brought about maturation of the cells into endothelial cells, producing von Willebrand factor (vWF) and with high lipoprotein metabolism. Up-regulation of the expression of SMC markers was achieved once supplemented with human PDGF-BB. In addition, colony formation unit assays revealed the progenitor populations' ability to form different hematopoietic colonies (Figure 27-5). Formation of tube-like structures and sprouting were observed, after seeding the differentiated cells within 3D collagen and Matrigel gels (Figure 27-6). Histological and electron microscopic examination showed the formation of a typical tube-like network and penetration of endothelial cells into the Matrigel. Derivations of endothelial sub-populations from Ebs, or blast-forming-cell-derived common progenitors, are based on cell sorting by using the expression patterns of multiple markers. The usage of a complex multi-step induction system of hESC differentiation, based on dynamic exchange of growth factors, for instance, results in a dynamic expression of the various markers. A sorted out $KDR^{low}c\text{-}kit^{neg}$ sub-population with cardiovascular potential gave rise to endothelial cells in the presence of bFGF.

Another approach to the enhancement of endothelial frequency in maturing hESCs is based on co-culture of these cells on murine bone marrow-derived feeders. Increased angiohematopoietic cell differentiation of hESCs was detected in the presence of bone marrow-derived stromal cell line OP9, and to a lesser extent with other murine bone marrow cell lines S17 and MS-5, or yolk sac endothelial cell line c166. OP9-derived sub-populations of either CD34+ or VEGFR2+ (KDR/flk) cells are then sorted for further enrichment in culture, or tested in various transplantation assays (e.g., Matrigel plug assay, hind limb ischemia model) for morphology analyses and vasculogenesis evaluations. In efforts to understand which stimuli are required for endothelial differentiation, two factors have been described so far: VEGF and mechanical forces such as shear stress. Both have been shown to activate the Flk-1-PI3K-Akt signaling pathway. However, it is still difficult to obtain a large number of endothelial cells with high purity for cell transplantation studies and characterization.

Although significant progress in our understanding of human developmental vasculogenesis has been achieved so far, via studies of vascular remodeling in human embryos as well as hESC-derived endothelial cells, further basic topics still need to be addressed: regulation and mechanisms of differentiation, potential induction in culture, efficient scaling-up procedures, and immunologic studies of hESC-derived ECs for clinical prevention and therapy of vascular disorders based on cell transplantation, or tissue engineering strategies.

ACKNOWLEDGMENTS

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KEY WORDS

Angiogenesis Maturation and remodeling of the primitive vascular plexus into a complex network of large and small vessels.

Cardiovascular progenitor A precursor cell that gives rise to cariomyocytes, endothelial and smooth muscle cells.

Embryonic stem cells Pluripotent cells, derived from the inner cell mass preimplanted blastocytes. Capable of unlimited proliferation under specific culture conditions.

Embryoid bodies Aggregates of spontaneously differentiating embryonic stem cells that recapitulate aspects of early embryonic development.

Hemangioblast/Hemogenic endothelium A precursor cell that gives rise to endothelial and hematopoietic cells.

Mesoderm The middle embryonic germ layer, between the ectoderm and endoderm, from which connective tissue, muscle, bone, cartilage, and blood vessels develop.

Vasculogenesis Generation of new blood vessels in which endothelial cell precursors undergo differentiation, expansion, and coalescence to form a network of primitive tubules.

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Chapter 28

Multipotent Adult Progenitor Cells

Catherine M. Verfaillie and Annelies Crabbe

INTRODUCTION

In this chapter, we want to provide updated information regarding a rare cell population we have named multipotent adult progenitor cells, or MAPC. In 2001–2002, we published a series of papers demonstrating that, while attempting to select and culture mesenchymal stem cells (MSC) from human and subsequently mouse and rat bone marrow (BM), we accidentally identified a rare population of cells that has characteristics unlike most adult somatic stem cells, in that they appear to proliferate without senescence, and have broad differentiation ability *in vitro* and *in vivo* (Reyes *et al.*, 2001; Jiang *et al.*, 2002).

ISOLATION AND PHENOTYPIC CHARACTERIZATION OF MULTIPOTENT ADULT PROGENITOR CELLS

MAPC can be cultured from mouse, rat, swine, and human bone marrow (BM). MAPC from all species are significantly smaller than their mesenchymal stem cell (MSC) counterpart, and differ phenotypically from MSC. We have phenotyped murine MAPC most extensively. Unlike MSC, murine MAPC do not express major histocompatibiliy (MHC)-class I antigens, do not express or express only low levels of the CD44 antigen, and do not express CD34 (Jiang *et al.*, 2002; Breyer *et al.*, 2006; Ulloya-Montoya *et al.*, 2007). Unlike hematopoietic stem cells (HSC), murine MAPC do not express CD45 and CD34, but like HSC, mouse MAPC express high levels of cKit. Unlike MSC, MAPC also do not express the PDGF-Rβ, but express PDGF-Rα (Ulloya-Montoya *et al.*, 2007). Human MAPC are HLA-class one low, do not express CD45 and CD34, but express low levels of CD133 (Aranguren *et al.*, 2007).

Unlike most adult somatic stem cells, MAPC proliferate without obvious signs of senescence, and have active telomerase. In humans, the length of MAPC telomeres is 3–5-kB longer than in neutrophils and lymphocytes, and telomere length is no different when MAPC are derived from young or old donors. This suggests that MAPC are derived from a population of cells that either has active telomerase *in vivo*, or that is highly quiescent *in vivo*, and therefore have not yet incurred telomere shortening *in vivo*. Human MAPC that are expanded to >70 population doublings, and hence well beyond the Hayflick point (Shay and Wright, 2000), remain cytogenetically normal. Likewise, MAPC isolated from swine bone marrow can be expanded to more than 80–100 population doublings without cytogenetic abnormalities, even though some cytogenetic abnormalities are seen when cells continue to be expanded for a longer period of time (Zeng *et al.*, 2006). Rodent MAPC can be culture-expanded for several hundreds of population doublings. However, after many rounds of cryopreservation and expansion, several populations of mouse and rat MAPC have acquired numerical, as well as structural, chromosomal abnormalities (manuscript in preparation). Such abnormalities are, however, not present in early passage cells (manuscript in preparation). We have not found significant cytogenetic abnormalities in human cell populations. The phenomenon of acquisition of cytogenetic abnormalities, more pronounced perhaps in rodent compared to larger mammalian cell populations, is a common problem seen when stem cells are expanded long-term *in vitro* (Rosler *et al.*, 2004; Maitra *et al.*, 2005; Mitalipova *et al.*, 2005; Bochkov *et al.*, 2007) and is a reason why many believe that safe clinical application of *ex vivo* expanded stem cells will likely require earlier passage cells to be used.

Although isolation of MAPC remains technically demanding, the maintenance of established cell lines in their undifferentiated form has become significantly less demanding. The challenge in MAPC isolation is that cells with morphological and phenotypic features of MAPC can only be detected in bone marrow cultures after several population doublings. As will be discussed below, we still have not yet determined whether MAPC exist as such in the freshly isolated bone marrow, or whether this cell phenotype

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is acquired via the culture process. Culture conditions that affect isolation of MAPC include cell density, CO_2, and O_2, and cell density, among others. An update on the culture conditions and quality control of rodent MAPC can be found in the manuscript by Breyer *et al.* (2006). A similar report will be forthcoming for the isolation and maintenance of human cells. Because of these optimizations, new investigators unfamiliar with the technology can now maintain the different cell lines with minimal instruction.

We have evidence that, under the same culture conditions, clonal populations can be isolated that differ in relation to cell phenotypic and expressed gene profile. These differences correlate with the breath of differentiation that can be observed for the different cell populations, as discussed in Ulloa *et al.* (2007). For instance, we have described that multiple murine cell populations isolated from a single bone marrow, or from different bone marrow samples, differ in the level of Oct4 expression, ranging from no Oct4 at all (mClone-1) (Ulloa *et al.*, 2007), to levels of Oct4 as high as 10% of that of murine ESC (qRT-PCR compared with the mRNA level of GAPDH: some clones express Oct4 at a delta CT of 4–8, with ESC expressing Oct4 at a delta CT of 2–3 (Ulloa *et al.*, 2007)). Likewise, when isolating clonal populations of rat MAPC, we have found cell populations without Oct4 (rClone-1) (Ulloa *et al.*, 2007), and populations with Oct4 mRNA at a delta CT compared with the household gene GAPDH of 2 (rMAPC-1 (Ulloa *et al.*, 2007), and several newer isolations).

Cell populations that do not express Oct4 cluster more closely by transcriptome analysis to MSC, whereas cell populations expressing higher levels of Oct4 cluster more closely with ESC. The expression level of Oct4 is also associated with differences in differentiation potential of the clonal cell lines: cells that do not express Oct4 differentiate significantly less well into cells with endothelial, endodermal, and neuroectodermal characteristics.

MAPC that express Oct4 also express a number of other transcription factors known to be necessary for the pluripotency of ESC, such as Sall-4 (Zhang *et al.*, 2006), Rex-1 (Cenki *et al.*, 2003), Tbx-3 (Rowley *et al.*, 2004), among others, or necessary for the induction of a pluripotent state in adult cells, such as Lin28 (Viswanathan *et al.*, 2008) or nMYC (Cartwright *et al.*, 2005). Noteworthy, MAPC that express Oct4 do not express Sox2 (Lewitzky and Yamanaka, 2007) and Nanog (Pan and Thomson, 2007), the two other key transcription factors involved in a recently well-characterized feed-back transcription system that maintains ESC undifferentiated (Chew *et al.*, 2005; Okita and Yamanaka, 2006). However, MAPC express several transcription factors involved in mesodermal and endodermal differentiation, including Gata-4 and Gata-6 (Fletcher *et al.*, 2006), myocardin (Wang and Olson, 2004), Pitx2 (Franco and Campione, 2003), Foxa2 (Friedman and Kaestner, 2006), and Sox17 (Sinner *et al.*, 2004), among others. This may suggest that MAPC can be placed developmentally between the epiblast stage and a primitive streak/mesendoderm committed cell type, which is perhaps consistent with the differentiation ability they exhibit (Figure 28-1).

IN VITRO DIFFERENTIATION POTENTIAL OF MULTIPOTENT ADULT PROGENITOR CELLS

MAPC from all species can be successfully differentiated into typical mesenchymal lineage cells, including osteoblasts, chondroblasts, and adipocytes. In contrast to MSC, human, swine, mouse, and rat MAPC can be induced to differentiate into cells with morphological, phenotypic and functional characteristics of endothelial cells (EC) (Jiang *et al.*, 2002; Ross *et al.*, 2006). Moreover, like ESC but unlike

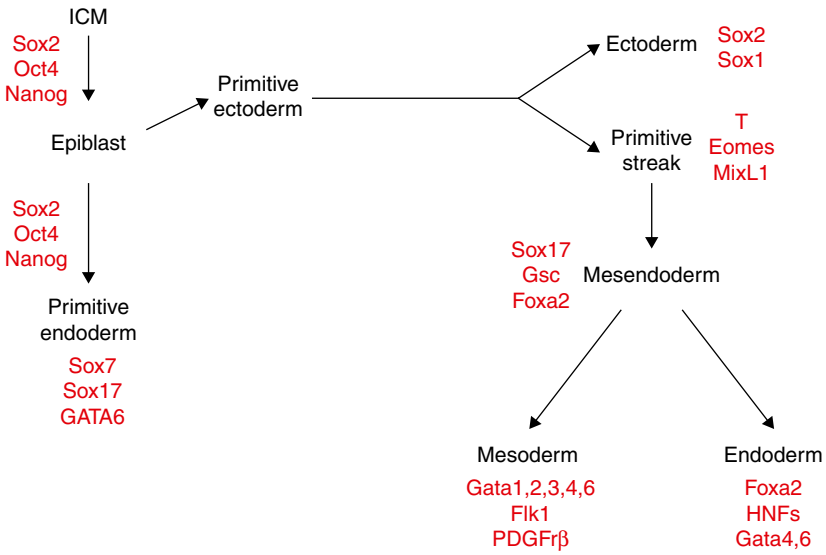


FIGURE 28-1 MAPC appear to have a phenotype consistent with a developmental stage between epiblast and mesendoderm/endoderm-mesoderm.

cord blood or bone marrow-derived CD133 cells in human, we have demonstrated that human MAPC treated with vascular endothelial growth factor (VEGF) start to express arterial and venous endothelium specific transcripts and proteins, and that addition of the Notch ligand, delta-like-4 (Dll4) and sonic hedgehog (Shh) inhibits venous, while enhancing arterial, endothelium specification (Aranguren *et al.*, 2007). This is true not only *in vitro*, but also when human MAPC are implanted in Matrigel *in vivo*.

MAPC from all species can be coaxed to differentiate into cells with phenotypic and functional characteristics of smooth muscle cells (SMC) (Ross *et al.*, 2006). Like primary aorta SMC from newborn rats, MAPC-SMC exhibit functional calcium channels. When entrapped in fibrin, in circular vascular molds, MAPC-SMC circumferentially align to generate ring-shaped constructs. Like constructs generated with primary SMC, MAPC-SMC derived constructs generate force in response to KCl, as well as a number of other agonists, including the L-type channel opener FLP64176, serotonin or endothelin-1, and complete relaxation when Rho-Kinase is inhibited.

We have published that human, mouse, rat, and swine MAPC can be committed down an endodermal path, more specifically giving rise to cells that have morphological, phenotypic and functional characteristics of hepatocytes (Jiang *et al.*, 2002; Zeng *et al.*, 2006; Ross *et al.*, 2006). In the initial studies we demonstrated that this could be accomplished by addition of high concentrations of dexamethasone and the cytokines, hepatocyte growth factor (HGF), and fibroblast growth factor-4 (FGF-4). Subsequent studies by Snykers *et al.* (2006) demonstrated that when the cytokines are added sequentially rather than simultaneously, more robust differentiation of MAPC to hepatocyte-like cells could be found. The same cytokines, HGF and FGF4, also induce human ESC towards cells with characteristics of hepatocyte-like cells (Schwartz *et al.*, 2005), although the percentage of albumin positive cells was low, and differentiation required initial culture in embryoid bodies (EB) (Schwartz *et al.*, 2005). Since the initial description of hepatic-like cell differentiation from cells with greater potency, we have further optimized the differentiation schema, and have developed a four-step protocol that allows not only ESC, but also MAPC to differentiate sequentially to mesendoderm/definitive endoderm—and this following expression of genes found during primitive streak formation, hepatoblasts, and ultimately hepatocyte-like cells. For ESC, differentiation can be obtained without intermediate EB formation. Progeny from such differentiation cultures is still impure, with 10–20% of cells expressing mature hepatocyte proteins, with persistent immature hepatoblasts, as well as continued presence of mesodermal cells, and undifferentiated ESC and MAPC. Nevertheless, functional studies have demonstrated that even such a mixed cell population contains a significant number of cells that display many functional features of mature hepatocytes (manuscript in preparation).

Finally, in 2003 we published that rodent MAPC can be induced down a neuroectodermal path, generating neurons expressing dopamine and with functional voltage-gated sodium channels. Since then we have continued to evaluate whether, like ESC, MAPC could be induced to commit to a neural stem cell (NSC) fate using methods described for ESC. Preliminary studies demonstrate that a neural stem cell phenotype (based on transcriptome and protein analysis) can be obtained from MAPC with culture conditions described for ESC (Conti *et al.*, 2005).

Interestingly, the lineage that continues to be elusive is cardiac myoblasts, a differentiation path that is almost a default differentiation pathway for mouse ESC. Although we have shown that expression of the transcription factors Nkx2.5, Tbx5, Mef2C, more mature cardiac structural proteins such as troponins, and myosin heavy chain mRNA and proteins can be induced, we have not observed spontaneous rhythmic contracting or beating cell populations. As we have solid evidence that MAPC can generate many types of mesodermal cells, as well as hepatocyte-like cells, following well-known developmental intermediates, the lack of differentiation to cardiomyocytes remains unexplained.

IN VIVO ENGRAFTMENT OF MULTIPOTENT ADULT PROGENITOR CELLS

In the initial description of murine MAPC in 2002, we demonstrated that when undifferentiated murine MAPC were transplanted intravenously in minimally irradiated murine hosts, low levels of engraftment within the hematopoietic system could be detected, and some engraftment was seen in the liver, lung and gastrointestinal epithelium (Jiang *et al.*, 2002). Since these initial studies, many more studies have been done to assess the ability of undifferentiated rodent, swine, and human MAPC to contribute to tissue formation/repair *in vivo*.

MULTIPOTENT ADULT PROGENITOR CELLS IN ISCHEMIA MODELS

Peripheral Vascular Disease

MAPC were injected in a mouse model of limb ischemia (Aranguren *et al.*, 2008). Murine undifferentiated MAPC, or a combination of MAPC-derived EC and MAPC-derived SMC, were compared with mouse bone marrow cells. Human MAPC were compared with human CD133+ cells from bone marrow or cord blood. Significant and persistent improvement in limb perfusion and muscle function were seen with undifferentiated MAPC, which outclassed human CD133+ cells and murine MAPC-EC/MAPC-MSC. Of note, murine BM cells after an initial improvement in perfusion and limb function, caused deterioration in the vascular status, as well as function of the limb, for reasons that are

not fully understood. The mechanisms underlying the effect of MAPC are likely mixed; the undifferentiated MAPC contribute, albeit relatively modestly, to endothelium, smooth muscle, and skeletal muscle. However, the number and size of host blood vessels was significantly increased following MAPC therapy, with decreased fibrosis, necrosis and significant increase in host myogenesis.

Cardiac Ischemia

In a second series of studies, rodent and swine MAPC were injected in the peri-infarct zone of the heart following left anterior descendent (LAD) artery ligation. Compared to BM mononuclear cells and fibroblasts, murine MAPC improved LV contractile function significantly for at least eight weeks post-transplantation, with a significantly greater decrease in scar size. MAPC, bone marrow cells or fibroblasts were present beyond one week; therefore, the effect of MAPC was not by differentiation and direct contribution to the vascular or cardiomyocyte compartment (Pelacho *et al.*, 2007). However, there was significantly greater vascularity eight weeks post-transplantation, possibly due to the production of monocyte chemoattractant protein-1 (MCP)-1, VEGF, platelet derived growth factor (PDGF)-BB, and transforming growth factor (TGF)-β_1, secreted by MAPC. Tolar *et al.* (2007) also grafted MAPC in the peri-infarct zone of murine hearts, using both immunocompetent, syngeneic C67Bl/6 mice and Rag2/IL-2Rγc$-/-$ immunodeficient animals. Significantly more MAPC could be detected in the heart of Rag2/IL-2Rγc$-/-$ than C57Bl/6 mice. However, left ventricular contractile function was significantly better in C57BL/6 than Rag2/IL-2Rγc$-/-$ recipients, likely due to increased vascular density in infarct and peri-infarct zones. Thus, the immune competence of the recipient appears to modulate the therapeutic impact of MAPC cells in myocardial infarcts, and a more vigorous immune response may be advantageous for therapeutic myocardial repair. In a third rodent study, Agbulut *et al.* (2006) grafted human MAPC in a rat model of chronic myocardial infarction. At one month the left ventricular function of untreated rats deteriorated significantly. Although the ejection fraction of cell treated animals remained more stable, the difference did not reach statistical significance. While MAPCs could be identified early post-transplant, no evidence of engraftment was further observed at one month by immunohistochemistry, electron microscopy, or PCR. Hence, in this model, MAPC did not improve global pump function, and although some of these cells expressed endothelial markers during the early post-transplant period, MAPC did not differentiate into cardiomyocytes.

In another study, swine MAPC were grafted in the peri-infarct zone of the heart of allogeneic swine. Some animals were treated with cyclosporine, whereas others did not receive immunosuppression (Zeng *et al.*, 2007). The beneficial effect of the MAPC was seen irrespective of the immunosuppressive therapy. Again, few cells persisted in the heart, but they induced significant neovascularization and cardiomyocyte regeneration that stabilized bioenergetic and contractile function in the infarct zone and border zone. Magnetic resonance imaging (MRI) demonstrated that the infarct zone systolic thickening fraction improved significantly from systolic "bulging" in untreated animals to active thickening, while the left ventricular ejection fraction improved significantly. This study thus demonstrates a possible role for allogeneic MAPC in the treatment of ischemic disease.

Stroke

A similar trophic effect as shown in peripheral vascular disease models, as well as cardiac ischemia models, was also seen when human MAPC were grafted in the penumbra of stroke in cyclosporine treated rats that underwent a middle cerebral artery occlusion (MCAO) seven days earlier. Although significant functional improvement was seen on week six, no significant contribution of the grafted cells to the vasculature or the brain parenchyma was seen; whether cyclosporine is needed to detect the beneficial effect was not tested (Zhao *et al.*, 2003).

In a second study, Yasuhara *et al.* (2006) tested the effect of syngeneic or allogeneic rat MAPC injected in the hippocampus of seven-day-old rats subjected to unilateral hypoxic-ischemic brain injury by ligation of the common carotid artery and subsequent exposure to hypoxic environment. All animals received immunosuppression. There was a significant reduction in motor asymmetry in MAPC-treated animals, and MAPC-treated animals could stay significantly longer on the rotarod, whether syngeneic or allogeneic cells were grafted. Although some cells persisted, again no significant differentiation towards neurons was seen. In a subsequent study, similar results were also seen when MAPC were transplanted systemically, compared with direct intrabrain transplantation (Yasuhara *et al.*, 2008).

SYSTEMIC TRANSPLANTATION OF MULTIPOTENT ADULT PROGENITOR CELLS

Tolar *et al.* (2006) demonstrated that MAPC which express low levels of major histocompatibility (MHC)-class I molecules do not stimulate T-cell alloresponses, and are targets of NK cytolysis. Following transplantation *in vivo*, MAPC thus persist best in animals devoid of NK cells. Based on this data, many of the studies, including transplantation of rodent MAPC in the ischemic limb model and myocardial infarct model described above, as well as the studies evaluating the differentiation of MAPC after systemic transplantation, were done using animals

in whom NK cells were eliminated by anti-asialo-GM1 antibodies.

When murine MAPC from CD45.2/GFP donor mice were intravenously injected into sub-lethally irradiated NOD–SCID mice also treated with anti-asialo-GM1 antibodies, MAPC-derived CD45.2+ donor cells could be detected in the blood, bone marrow, and spleen at 10–20 weeks after transplantation (Serafini *et al.*, 2007). However, MAPC could not rescue animals from radiation-induced pancytopenia. MAPC generated 1–2% ckit+ Sca1+ Lin− (KSL cells) cells, indicating that they can generate LT-HSC. This was confirmed by transplantation of Lin− cells, as well as KSL cells, in lethally-irradiated secondary recipients, where they could re-establish long-term hematopoiesis.

Chimera Formation

Finally, we have shown that transfer of 10–12 mouse MAPC into mouse blastocysts results in the generation of chimeric mice, with the degree of chimerism varying between 1–40% in about 50% of mice (Jiang *et al.*, 2002). When more recently derived MAPC lines were used, significantly less chimerism was seen, with a maximal 2% degree of chimerism. The reason for the differences between lines is not known. Also not known is whether the initial high levels of chimerism seen in rare animals is due to fusion between MAPC and cells within the inner cell mass, or via direct contribution. Also not yet known is whether MAPC contribute to the germline when injected in the blastocyst.

OTHER ADULT STEM CELLS WITH GREATER DIFFERENTIATION POTENTIAL

Since our publication of 2002, many other groups have isolated cells from bone marrow, cord blood, and amniotic fluid with characteristics similar to those of MAPC. Specifically bone marrow-derived multipotent stem cells (BMSC) (Yoon *et al.*, 2005), marrow-isolated adult multilineage inducible cells (MIAMI) (D'Ippolito *et al.*, 2004), pre-mesenchymal stem cells (Anjos-Afonso and Bonnet, 2007), very small embryonic-like cells (VSEL) (Kucia *et al.*, 2006), and multipotent adult stem cells (MASC) (Beltrami *et al.*, 2007) have been isolated from bone marrow of mouse and man, amniotic fluid derived stem (AFS) cells are isolated from amniotic fluid (De Coppi *et al.*, 2007), and unrestricted somatic stem cells (USSC) come from umbilical cord blood (Kogler *et al.*, 2006). All these cell populations are CD45−ve cells with some degree of variation in expression of cell surface antigens. They can be cultured for extended periods, and differentiate to cells of at least two different germlayers. Some of these cell populations may express the ESC-specific transcription factors, Oct4, Sox2, and Nanog (Lewitzy and Yamanaka, 2007; Pan and Thomson, 2007; Niwa *et al.*, 2000). It should, however, be noted that for human cells, care needs to be taken when assessing Oct4 and Nanog expression, as multiple pseudogenes exist for these transcription factors, making RT-PCR evaluation technically difficult (Liedtke *et al.*, 2008). An overview of the different adult stem cells with greater differentiation potential can be seen in Table 28-1.

POSSIBLE MECHANISMS UNDERLYING THE GREATER POTENCY OF MULTIPOTENT ADULT PROGENITOR CELLS

Currently we do not fully understand the mechanism(s) underlying the greater potency of MAPC. We have definitive data to demonstrate that the greater potency of MAPC is not due to presence of multiple stem cells within the cultured MAPC population. Using retroviral marking studies, we have shown that a single cell can differentiate *in vitro* to cells of mesoderm, both mesenchymal and non-mesenchymal, neuroectoderm, and hepatocyte-like cells (Jiang *et al.*, 2002).

A second possibility for the greater degree of differentiation potential would be that cells undergo fusion and acquire greater pluripotency via this mechanism. Fusion has been shown to be responsible for apparent ES characteristics of marrow and neural stem cells that had been co-cultured with ES cells *in vitro*, and for the apparent lineage switch of bone marrow cells to hepatocytes, neural cells, and cardiac and skeletal muscle cells. In the ESC-neural/hematopoietic cell fusions occurring *in vitro* (Ying *et al.*, 2002; Terada *et al.*, 2002), the majority of genes expressed in the marrow or neural cell that fused with ESC were silenced, and the majority of the genes expressed in ESC were persistently expressed. Likewise for the bone marrow-hepatocyte fusion occurring when hematopoietic cells were infused in animals with hereditary tyrosinemia due to lack of the fumarylacetoacetate hydroxylase (FAH) gene, the majority of genes expressed normally in hematopoietic cells (except the FAH gene) were silenced, whereas gene expressed hepatocytes predominated (Dorrell and Grompe, 2005; Camargo *et al.*, 2004; Wang *et al.*, 2003). The fused cells were in general tetraploid or aneuploid. It is unlikely that this phenomenon underlies the greater potency of MAPC, as cultivation and differentiation *in vitro* does not require that MAPC are co-cultured with other cells, making the likelihood that MAPC are the result of fusion very low. Smith *et al.* suggested in a commentary that MAPC could be caused by fusion of multiple cell types early on during culture leading to reprogramming of the genetic information and pluripotency. However, we have no evidence that MAPC, even early during culture, are tetraploid or aneuploid, making this possibility less likely.

TABLE 28-1 An Overview of Different Adult Stem Cells with Greater Differentiation Potential

Name	Source	Phenotype	ES transcription factors	Differentiation capacity
MAPC (Jiang *et al.*, 2002)	Bone marrow	Mouse: CD45−, PDGFRα+, ckit+, Sca1^{low} Rat: CD45−, CD44^{dim}, CD31+ Human: CD45−, CD44+, CD105−, CD90+, MHC−I^{dim} Swine: CD45− CD45−, MHC−	Oct4+, Nanog− Sox2−	Mesoderm, endoderm, and neuroectoderm-like cells Some mouse lines contribute to chimeric animals
BMSC (Yoon *et al.*, 2005)	Human bone marrow	CD29−, CD44−, CD73−, CD105−, CD90−, CD117−	Oct4−	Mesoderm, endoderm, and neuroectoderm-like cells
MIAMI (D'Ippolito *et al.*, 2004)	Bone marrow	CD34−, CD36−, CD45−, CD117−, HLA−, CD29+, CD63+, CD81+, CD122+, CD164+	Oct4+, Rex1+	Mesoderm, endoderm, and neuroectoderm-like cells
Pre-MSC (Anjos-Afonso & Bonnet, 2007)	Murine bone marrow	Lin−, CD45−, CD31− or CD45−, CD11− and SSEA1+	Oct4+, Nanog+, Sox2+	Mesoderm, endoderm, and neuroectoderm-like cells
VSEL Kucia *et al.*, 2006)	Human and mouse bone marrow/umbilical cord blood	Sca1+, lin−, CD45−	Oct4+, Nanog+, Sox2+	Mesoderm, endoderm, and neuroectoderm-like cells
AFS (De Coppi *et al.*, 2007)	Human amniotic fluid	CD45−, CD34−, CD133+, CD29+, CD44+, CD73+, CD90+, CD106+, MHCI+	Oct4+	Mesoderm, endoderm, and neuroectoderm-like cells
USSC (Kogler *et al.*, 2006)	Human umbilical cord blood	CD14−, CD33−, CD34−, CD45−, CD49−, CD50−, CD62−, CD106−, CD117−, CD13+, CD29+, CD44+, CD90+, CD105+	Oct4−, Nanog−	Mesoderm, endoderm, and neuroectoderm-like cells
MASC (Beltrami *et al.*, 2007)	Human bone marrow Liver Heart	CD13+, CD49b+, CD90+, CD73−, CD44−, HLA−, CD29−, CD105−, CD49a−	Oct4+, Rex1+, Nanog+	Mesoderm, endoderm, and neuroectoderm-like cells
Germ cells (Kanatsu-Shinohara *et al.*, 2004)	Mouse testis	SSEA1+, EE2+, CD9+, integrin+, EpCAM+, ALP+	Oct4+, Rex1+, Nanog+	Embryoid bodies and teratomas
MASC (Seandel *et al.*, 2007)	Mouse testis	GPR125+, ckit+, PLZF−, DAZL−	Oct4+, Nanog+, Sox2+	Embryoid bodies, teratomas, and partial chimeric mice
maGSC (Guan *et al.*, 2006)	Testis	Sca1^{dim}, Ter119−, CD34−	Oct4+, Nanog+, Rex1+	Mesoderm, endoderm, and neuroectoderm-like cells Embryoid body formation Teratoma formation Chimeric mice, including germline

The *in vivo* studies were not set up to rule out this possibility fully. As indicated above, it remains possible that the chimera formation seen with some of the MAPC lines might be due to fusion of MAPC with cells in the inner cell mass (Jiang *et al.*, 2002). The contribution of MAPC to skeletal muscle in the limb ischemia model is certainly due to fusion, as muscle fibers are the result of fusion events (Aranguren *et al.*, 2008). Whether MAPC also generate satellite cells, the muscle fiber progenitor, was not addressed. We do, however, have evidence that skeletal muscle can be generated from mouse and human MAPC *in vitro*, without the need for cell fusion (Araguren *et al.*, 2008; Muguruma *et al.*, 2003). When injected in ischemic swine hearts, rare cardiac muscle cells, apparently derived from donor MAPC, were seen (Zeng *et al.*, 2007). Likewise, when murine MAPC were grafted intravenously in sublethally irradiated mice, rare apparently donor-derived cardiomyocytes were seen (Serafini *et al.*, 2007). However, the studies did not allow us to determine whether this is due to fusion or not. The contribution of MAPC to the hematopoietic system is, however, unlikely due to fusion, as no cells could be found that co-expressed markers from host and donor origin, and cytogenetic analysis demonstrated that the MAPC-derived hematopoietic cells were euploid (Serafini *et al.*, 2007).

Currently, we do not know whether MAPC exist *in vivo*, or are a culture-induced phenomenon. Several approaches have been used to isolate cells from murine or rat bone marrow or brain with a phenotype of culture established MAPC, including expression of SSEA1, c-Kit, CD31, PDGFRα, but absence of CD45 or Sca1, and presence of Oct4 (using Oct4-GFP transgenic mice obtained from The Jackson Laboratory or Oct4-knockin add-on GFP mice, a kind gift from Dr R. Jaenisch) (Lengner *et al.*, 2007). No cells with the phenotype or morphology of MAPCs could be isolated. During MAPC isolation, cells are maintained for three to four weeks prior to subcloning at five cells following column depletion of CD45+/Ter119+ cells. If MAPC are present in fresh bone marrow, cells proliferating from the five-cell subcloning step would contain 20% MAPC. However, we see the appearance of small cells that express Oct4 and that proliferate relatively rapid out-growing the rest of the culture only after six to eight weeks following subcloning. This suggests strongly that MAPC may not exist in rodent tissues, but are in part generated in culture. This notion is consistent with the data from Afonso *et al.* (2007), who demonstrated that rare cells in the bone marrow of mice expressing SSEA1 and very low levels of Oct4 mRNA can, under MAPC culture conditions, be expanded *in vitro* with a 100–10 000-fold further induction of Oct4 transcript levels following culture. It should be noted that the low levels of Oct4 expression in the freshly-isolated cells was quickly lost when cells were cultured under MSC conditions, and that cells that did not express SSEA1 or Ocr4 could not be induced to express these markers, even when cultured under MAPC conditions. These studies may thus suggest that MAPC are generated from a rare sub-population of cells within bone marrow under the specific culture conditions used to isolate and expand the cell population.

Consistent with the notion that culture may induce a more immature phenotype are studies from three groups demonstrating that when spermatogonial stem cells are cultured *ex vivo*, cell populations are established that have nearly all or all characteristics of ESC, such as expression of Nanog and Sox2 after two weeks to two to three months, aside from Oct4, differentiation to embryoid bodies, and for one of the cell populations contribution to all tissues, including the germline, in chimeric mice. These cells were named multipotent adult stem cells or MASC, and multipotent/adult germline stem cells or maGSC (Beltrami *et al.*, 2007; Kanatsu-Shinohara *et al.*, 2004; Guan *et al.*, 2006).

This notion is obviously also supported by the recent findings by, initially, the group of Dr S. Yamanaka (Takahashi and Yamanaka, 2006), and since then by many other groups (Okita *et al.*, 2007; Wernig *et al.*, 2007; Yu *et al.*, 2007; Lowry *et al.*, 2008; Park *et al.*, 2008), that it is possible to reprogram mouse and human terminally differentiated cells to an embryonic-like cell, called induced pluripotent stem cells, following the overexpression of Oct4 and Sox2, alone or combined with either Klf4, c-Myc, Nanog, and/or Lin28. There is already evidence that transduction with transcription factors may not be necessary if the same signaling pathways can be activated using small molecules (Shi *et al.*, 2008). Hence, we hypothesize that culture of bone marrow cells, under stringent MAPC conditions, may lead to the reprogramming of certain cells in the marrow to a MAPC-like state. It will obviously be of importance to understand the mechanism(s) underlying the putative spontaneous reprogramming to enhance the efficiency of this phenomenon.

KEY WORDS

Adult stem cell Cell derived from postembryonic stage tissue with self-renewal ability, and ability to generate all cell types of the tissue where it has been derived from, but not other tissues.

MAPC (multipotent adult progenitor cell) Cell isolated from murine bone marrow and created in culture, able to self-renew and generate certain cell types (e.g., endoderm, mesoderm).

Neural stem cell A type of an adult stem cell located in the brain. When there is an injury, these cells can move towards the site of injury, where some can replace a mature neuron, astrocyte or oligodendrocyte.

Trophic effect When stem cells are transplanted in a damaged tissue, they release certain growth factors and

chemokines in order to stimulate the endogenous stem cells to replace the lost cells.

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Chapter 29

Mesenchymal Stem Cells

Arnold I. Caplan

INTRODUCTION

In the mid-1980s, my laboratory started experiments to purify mesenchymal stem cells (MSCs) from adult human bone marrow. There were several reasons for initiating our approach, and for our success. It is important to briefly review these events because they established the basis for the current state of the field and the basis for interesting refinements, new observations, and new directions.

From 1968 to 1972, my laboratory developed and refined the methods for liberating and culturing undifferentiated mesenchymal cells from the developing limb buds of 4.5-day-old chick embryos. These embryonic chick limb bud cells multiplied in culture, and depending on the culture conditions and the initial seeding densities, they differentiated into muscle, cartilage, bone, and various connective tissues. At intermediate plating densities, bone-forming cells called osteoblasts were observed to develop, and at a 2.5-fold increase in initial plating density of these same cells, the plates were filled with cartilage with little or no evidence of osteoblast differentiation. The chondrocytes, which formed the cartilage tissue, were shown to continuously modulate the synthesis of the major cartilage water structuring molecule, a proteoglycan called aggrecan; these same changes have also been observed for human articular cartilage. Thus, these cell cultures mimic the full range of cartilage changes from young to old, from bouncy water-filled shock absorbers to the leathery, tough coverings of the joint surface.

In 1965, Professor Marshall R. Urist published his landmark paper on the purification of factors from demineralized bovine bone that induced cartilage and subsequent bone formation in various *in vivo* implantation systems and *in vitro*. The basis for Urist's assays was the observation that demineralized bone chips, when implanted into muscle or subcutaneous pouches in test animals, caused the formation of ectopic bone. Urist coined the term bone morphogenetic proteins (BMPs) to describe the presumptive inductive molecules that stimulated this tissue outcome.

In 1978, Glenn Syftestad joined my laboratory as a postdoc following a stay in Urist's laboratory. Syftestad was determined to purify BMPs from bovine bone, and the embryonic chick limb bud mesenchymal cell system was an obvious way to test for the chondrogenic stimulating activity (CSA) within the extract of demineralized bovine bone. The intermediate plating density where cartilage was never observed became the assay system for identifying and purifying chondrogenic inductive molecules from extracts of adult bone by observing chondrogenic differentiation under conditions in which no cartilage formed normally. Several papers and patents were published on these efforts and centered on a 31-kDa protein. We can now surmise that CSA was a heterodimer of BMPs; unfortunately, we, like Urist and his colleagues, were never able to sequence the proteins or to clone the genes. John Wozney and his collaborators at the Genetics Institute in Cambridge, Massachusetts (now part of Wyeth Pharmaceutical Company) were the first to do this.

In the course of studying CSA, we did many *in vivo* implantations, and observed ectopic cartilage and bone formation. During the course of these studies in the mid-1980s, it occurred to me that cells like the multipotent embryonic chick limb mesenchymal cells must reside in, or must come to, the implantation site in the adult tissue in order for cartilage and bone to form. I call these adult cells MSCs. By this time, several investigators had proposed that osteogenic (bone-forming) progenitor cells reside in bone marrow. This seemed obvious, since all bone is in contact with marrow elements. The experiments of Maureen Owen—and, in particular, her review article in 1988 that summarized A. J. Friedenstein's early work and included the possibility that fat cell progenitors were also in marrow—opened the door for speculation that all of the mesenchymal phenotypes could arise from this same class of progenitors in pathways analogous to those deduced for the blood-forming cells (hematopoiesis) (Figure 29-1).

In this context, several years earlier, my laboratory was deeply involved in the study of bone formation in the

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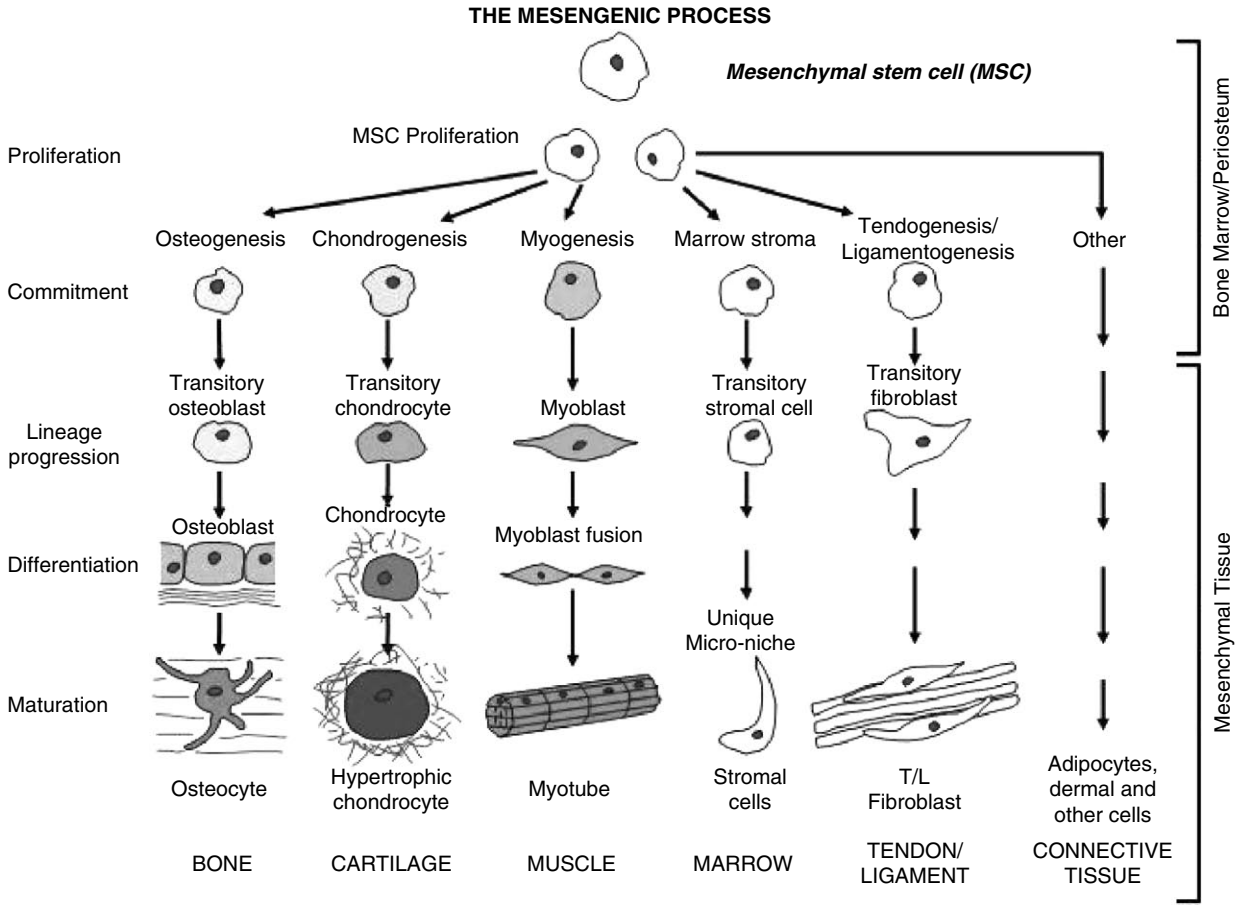


FIGURE 29-1 The mesengenic process depicts mesenchymal progenitor cells entering distinct lineage pathways that contribute to mature tissues.

embryonic chick limb bud *in vivo*, and in an attempt to compare it to *in vitro* events, a young and talented graduate student, Philip Osdoby, had wasted 18 months working with this limb bud mesenchymal cell culture system, because we followed the dogma of the day that cartilage was the progenitor of bone, as evidenced by endochondral bone formation and growth plate dynamics. We studied this by plating embryonic chick limb cells at high density to encourage them to form mounds of cartilage. No matter how we varied the culture medium or its additives, we could not get these cultures to form bone. Osdoby made a key observation in his superb electron micrographs of these cartilage cultures that when rare, mineralized deposits (osteoid) were observed, they were never located in the cartilage nodules, but always in the fibrous tissue between these massive mounds of cartilage. We then realized that osteoblasts formed independently of cartilage and away from it. By plating the cells at a density where cartilage never formed, we could observe many osteoblasts.

When we went back to the whole developing embryonic limb bud and looked at the location of first bone formation, it was outside and away from the already formed cartilaginous rods. Later, based on this early work of Osdoby and the subsequent morphological descriptions of first bone formation, Scott Bruder raised monoclonal antibodies to the tediously dissected first bone collars of the embryonic tibia and used these to establish the lineage transitions of mesenchymal progenitors to osteoblasts and osteocytes.

Thus, from this early work on embryonic mesenchymal cell differentiation, we had the osteogenic and chondrogenic lineages. Others had worked out many of the details of myogenic lineages, and quite interestingly, the suggestion had been made that hematopoietic support tissue (the marrow stroma) also contained cellular elements that provided differential support for separate arms of the hematopoietic pathway. The summary diagram in Figure 29-1 is therefore a composite of many individuals' efforts and was fashioned to mimic the fundamental aspects of the hematopoietic pathway; I called this multilineage pathway the "mesengenic process." I named the multipotent progenitor the mesenchymal stem cell, because all of the lineage pathways depicted in Figure 29-1 were mesenchymal in origin. The reasons for calling this cell a stem cell were rather loose, but were primarily inferred from the observation that the cells we isolated could be induced into different

lineage pathways. Thus, these "stemmed" from a common source.

Adult mesenchymal tissues all have unique turnover dynamics—some relatively rapid, like bone, and others relatively slow, like cartilage. In all cases, turnover means that differentiated cells in the tissue die and are replaced by newly created cells. For bone, this death and birth can take place when large sheets of osteoblasts are fabricating sheets of osteoid that will eventually mineralize. Several full generations of cells may come and go while a sector of bone is being fabricated.

Location of Mesenchymal Stem Cells

By the preceding logic, MSCs must occur everywhere mesenchymal tissue turns over. To date, mesenchymal progenitors have been isolated from marrow, muscle, fat, skin, cartilage, and bone. Moreover, every blood vessel in the body has mesenchymal cells on the tissue side of the vessel (some of these are MSCs), although names like pericytes have been given to these multipotent cells.

Test for Mesenchymal Stem Cells

The tests for MSCs are straightforward: populations of cells are placed in medium conditions that include exposure to strong phenotypic inducers. Following phenotypic induction, the expressional activity and morphology of the cells are monitored, and a judgment is made that a specific phenotype is present, based on the analysis of indicator molecules, morphologies, or both. Of course, *in vivo*, we have no unique markers for MSCs, although Stephen Haynesworth and I long ago isolated three monoclonal antibodies (SH2, SH3, and SH4) that preferentially bind to MSCs compared to hematopoietic cells.

Plasticity

One recent experimental complication is that we can take mesenchymal cells of a known phenotype, for example, an adipocyte with fat droplets inside, and expose these cells to inductive medium for another phenotype, for example, osteoblasts. These newly induced cells stop producing fat-specific molecules and start producing bone-specific molecules in osteogenic inductive medium. The capacity to be induced from one lineage into a new lineage is referred to as "plasticity." Once this is recognized, the functional test for an MSC becomes less stringent, because non-MSCs, like the adipocytes cited previously, and MSCs can be induced to become osteoblasts. For emphasis, this is an experimental situation and may not be relevant to *in vivo* events. However, a cell's capacity for plasticity clouds the issue of identifying MSCs by these functional assays for multiple mesenchymal phenotypes.

Mesenchymal Stem Cells Decrease With Age

The body's need for MSCs changes dramatically, depending on the age of the individual. In embryos, mesenchymal tissues form from a relatively high local density of progenitors within a very loose, watery extracellular matrix. The massive differentiation of these groupings of progenitors into differentiated cells, and thus into tissues, involves the substantial up-regulation of synthesis of complex and tissue-specific extracellular matrices. The cell-to-matrix ratios go from high to low during these differentiation events. Moreover, the matrix adds to the many-fold increase in the gross size of the tissues being formed. In this process, many of the progenitor cells are directly converted to differentiated cells, and the relative MSC numbers dramatically decrease in the embryo. Similarly, from birth to the teens, the increase in body-part sizes is many-fold; this also must involve the direct conversion of some of the MSCs into differentiated phenotypes. In this regard, it seems reasonable to expect a mesenchymal tissue injury of a child below the age of 5 years to regeneratively repair because of relatively high MSC levels; in a 20-year-old, the same tissue might only repair with fibrous scar tissue. To take this comparison even further, it would seem reasonable to expect that 50-year-olds would have fewer MSCs locally than 20-year-olds to repair mesenchymal tissue injuries.

Since we have no unique probes for MSCs, we cannot determine the whole-body titers of MSCs. As an estimate, we can measure the colony-forming units—fibroblasts from marrow aspirates or marrow biopsies. Even in this assay, the differences in marrow aspiration techniques, medium additives, and growth-medium serum levels (and prescreening batches of serum) preclude establishing an experimental consensus on the absolute numbers of MSCs in marrow. What everyone seems to agree upon is the *decrease* of MSCs in marrow with age.

Based on this information, I propose six periods of differing MSC titers. Each period would be expected to have a different capacity for tissue expression and regenerative repair. The different periods are: (1) embryology, fertilization to birth; (2) neonatology, birth to 6 years; (3) teens, 7 to 20 years; (4) peak skeletal performance, 20 to 30 years; (5) midlife, 35 to 55 years; and (6) late life, 60 to 90 years. The important distinction is that, although HSC titers and potency are relatively constant from birth to late life, MSC titers change by one or two orders of magnitude. This highlights the fundamental issue that both the definitions and the functional biology of MSCs and HSCs are expected to be different.

TISSUE-ENGINEERED REGENERATION

With the preceding information as a base logic, it follows that the regenerative repair of certain mesenchymal tissues

will require the augmentation of MSC numbers at the repair sites in post-teen adults. This can be accomplished in several ways depending on the age and health status of the individual. For years surgeons have used freshly harvested whole marrow to augment local skeletal tissue healing, and methods to concentrate and enrich the marrow are being introduced commercially. Key to this latter use, and to the use of culture-expanded MSCs, are the type and properties of the scaffolds used to isolate, concentrate, and/or deliver the cells to the repair site. Such composite grafts form the backbone of the new scientific discipline of tissue engineering.

ISOLATING AND CULTURING MESENCHYMAL STEM CELLS

MSCs have been isolated from several species and tissues, but the most well characterized, and probably the purest preparation, is from human bone marrow. The studies to purify MSCs from human marrow were initiated in the mid-1980s by Stephen Haynesworth in my laboratory. The key to our initial and rapid success was the lot of fetal bovine serum used to initiate these studies. The original batch of serum was selected from several lots by a selection assay using embryonic chick limb bud mesodermal cells in culture. At that time, we would prepare such cultures weekly from 200 dozen fertile eggs; batches of serum varied greatly from the same commercial supplier and from many suppliers. Based on color alone, many batches, by experience, were not suitable. (A dark brown or greenish tint indicated hemolyzed blood, and batches with these colors were not suitable.) The serum accounts for three key aspects of the MSC purification–expansion protocol: (1) selective attachment to tissue culture plastic to provide discrete colonies of MSCs; (2) optimal mitotic expansion of the cells; and (3) maintenance of the undifferentiated or stem cell state of the MSCs.

Today we use a laborious derivative of our initial, published screening protocol. In this regard, it is more difficult to screen batches of serum in that only one in 20 to 30 lots is suitable for human MSCs. This is because some suppliers blend batches to optimize their use in hybridoma cultures, making it more difficult to screen for a suitable lot for MSCs. Moreover, every animal preparation requires its own lot of serum, with the batch for human being unsuitable for rat, rabbit, or mouse marrow MSCs. (In some cases, batches of serum optimal for human MSCs are toxic to mouse MSCs.) Thus, if an unscreened batch of serum is used, or if an agent such as dexamethasone is added to the basic growth medium, results for colony counts and for cellular response profiles to other bioactive agents will differ from those obtained in my laboratory. In evaluating other reports using MSCs, information pertaining to the serum and other medium components is crucial.

In the early 1990s, Haynesworth, our colleagues, and I organized the transfer of the MSC technology from Case Western Reserve University to a newly formed company called Osiris Therapeutics, Inc. (OTI, now in Baltimore, Maryland). The scientists at OTI have repeated and refined our basic technology and have added to the database of human MSCs. They showed that by second passage the MSC preparation was homogeneous by fluorescence-activated cell-sorting criteria using more than 100 cell-surface antibodies and observed that clones could be obtained that showed multipotency for different phenotypic lineages. Our efforts to use mouse, rat, rabbit, dog, goat, and sheep marrow MSCs for tissue engineering were also transferred to OTI, where various preclinical animal models extended the results first obtained through my laboratory. OTI expanded the safety studies and initial human clinical trials started at Case Western Reserve University and University Hospitals of Cleveland.

TISSUE-ENGINEERING USES OF MESENCHYMAL STEM CELLS

In mature adults, marrow MSCs are used to supply progenitors for normal turnover and for repair–regeneration of tissue damage. The basis for this statement is the obvious involvement of MSCs in marrow stroma in support of normally active hematopoiesis, and of MSCs in normal bone turnover, to both directly support the formation of osteoblasts and indirectly support the formation of osteoclasts through two different lineage pathways (mesengenesis and hematogenesis, respectively). Thus, the use of culture-expanded MSCs to massively reform or repair certain tissues far exceeds their normal use and availability. In this case, we have adopted the logic that, to accomplish reconstruction of tissues that cannot normally themselves provide suitable numbers of progenitors, we must provide these progenitors from exogenous sources. Concomitantly, we must attempt to mimic selected principles of embryonic development and recapitulate these events in adults. The most important aspect of embryonic mesengenesis is that the progenitor cell to matrix ratio is very high just prior to lineage pathway entrance, and that the exact limits or edges of the neotissue are defined. Moreover, we now recognize that each site and each tissue have a unique sequence of inductive events and boundary conditions. Next, I briefly describe the use of MSCs for specific tissue regeneration, taking this embryonic recapitulation logic into account as depicted in Figure 29-2.

Bone Repair

The first and most obvious use of MSCs is in the area of bone regeneration in sites where the body cannot organize this activity, such as in nonunions. Critical size defects in

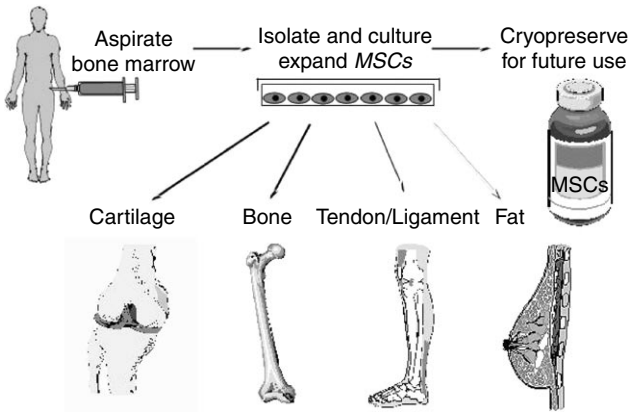


FIGURE 29-2 From an iliac bone marrow aspirate, MSCs could be used for tissue-engineered repair of cartilage, bone, or tendon or for plastic-surgical implant of fat from fresh or frozen stored cells.

nonunion models have shown that culture-expanded marrow MSCs in a porous, calcium phosphate, ceramic delivery vehicle are capable of regenerating structurally sound bone, where whole marrow or the vehicle alone cannot satisfactorily accomplish this repair. These preclinical models include the use of human MSCs in a femoral nonunion model in an immuno-compromised rat that will not reject the human cells.

Cartilage Repair

Cartilage is an avascular tissue incapable of regeneration–repair of even small defects in adults. Although chondrocytes have been used to attempt to repair large cartilage defects, it is difficult to integrate the neotissue with that of the host. In this case, we have pioneered the use of hyaluronan (HA) scaffolds, because of the high content of HA in the embryonic mesenchyme of precartilaginous tissues. These HA-based scaffolds are being tested in human clinical trials in Europe using autologous chondrocytes. These scaffolds provide an inductive microenvironment for MSCs to enter the chondrogenic lineage, and HA-scaffold breakdown products (oligomers) appear to facilitate the integration of neotissue with that of the host.

Marrow Regeneration

Injected MSCs make it back to the bone marrow to refabricate injured marrow stroma. This observation is the basis for a multi-location clinical trial to add back autologous MSCs to chemotherapy–radiation patients receiving bone marrow transplants.

Muscle Regeneration

We were the first to document that congenic (i.e., normal) MSCs could be injected into a specific muscle of the muscular dystrophy (*mdx*) mouse to cure it by providing newly synthesized dystrophin to the affected myotubes. In this case, the donor MSCs differentiated into skeletal myoblasts, fused with the host myotubes, and caused the synthesis and distribution of dystrophin. Moreover, labeled MSCs injected into injured (infarct model) rat or pig heart appear to differentiate into cardiac myocytes.

Fat

MSCs have been induced into the adipocyte pathway and can massively accumulate fat droplets. Although not used in tissue engineering models, we propose that bags of autologous fat are more appropriate for some plastic surgeries than bags of saline or silicone.

Tendon Repair

Studies initiated in my laboratory, and later completed at OTI and the University of Cincinnati, clearly establish the use of autologous MSCs for repair of tendons. The innovative design of an MSC-contracted collagen gel around a restorable suture held in tension allows tendon defects to be spanned by these composite constructs under normal loads. In this case, the same MSC able to differentiate into cartilage or bone develops into appropriate tendon tissue at Achilles or patellar tendon sites.

Gene Therapy

The studies with the *mdx* mouse cited previously establish the capacity of allo-MSCs to cure genetic defects. This principle has been translated to the clinical use of allogeneic bone marrow transplantation with or without additional culture-expanded MSCs to attempt to cure polysaccharide storage diseases or *Osteogenesis imperfecta* with substantial success. In addition, we long ago showed that human MSCs could be transfected with a retroviral construct without affecting their differentiation capacity. Molecules coded for by these gene inserts could be shown to be present many months after introduction into the *in vivo* model. Thus, MSCs hold the potential for a variety of gene therapy applications.

Vascular Support

The relationship between vascular endothelial cells and MSCs is not well studied. It is clear that multipotent mesenchymal progenitor cells are present on blood vessels (pericytes). More important are the details of the interaction between these vascular support cells and angiogenesis–vasculogenesis. Certainly, in embryonic angiogenesis, MSCs play a strong role. In adults, for example, it is now clear that vascular endothelial cells in bone make skeletal inductive molecules, such as BMPs. In addition, MSCs make vascular endothelial growth factor. Based on

these and other observations, we predict that, although nearly all tissues contain blood vessels, each tissue has specifically differentiated endothelial cells, and perhaps even unique MSC-derived vascular support cells.

SUMMARY

Adult bone marrow contains mesenchymal progenitor cells that may be called MSCs. Whether these preparations are composed of a pure population of stem cells or a spectrum of cells ranging from pluripotent stem cells to lineage-committed progenitors, it seems clear that these culture-expanded cells have the impressive potential to be used in tissue-engineered regeneration and repair of a variety of tissues. In some cases, as in bone nonunions, these cells are capable of differentiating directly into osteogenic cells. In the cases where they are used in cardiac (infarct) or brain (stroke) therapy, these cells appear to provide instructive and inductive microenvironments, inhibit scarring, and stimulate blood vessel regeneration for facilitated repair of critical lesions. That mesenchymal cells appear to be unusually plastic in their developmental capabilities opens the door for their use in several cell-based therapies.

ACKNOWLEDGMENTS

My thanks to my colleagues and collaborators at Case Western Reserve University and elsewhere for their assistance and encouragement. The National Institutes of Health generously supported much of the experimentation reviewed here.

KEY WORDS

Mesenchymal stem cells Cells that have the capacity to form different mesenchymal tissues given exposure to different inductive agents.

Mesenchyme The middle layer of an embryo, the mesoderm and the tissues that form (bone, cartilage, muscle, fat, etc.).

Osteoblast A cell formed by a multi-step differentiation sequence that becomes capable of fabricating bone.

Tissue engineering A new science that encompasses aspects of the natural and physical sciences that focus on the repair or regeneration of various tissues.

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Chapter 30

Skeletal Muscle Stem Cells

Helen M. Blau, Alessandra Sacco and Penney M. Gilbert

INTRODUCTION

In recent years, significant progress has been made in the study of tissue-specific adult skeletal muscle stem cells (MuSC) as novel strategies to prospectively isolate and transplant this stem cell population have been developed. Here we discuss the identification of diverse MuSCs in adult animals, their biochemical and functional heterogeneity, their regulation by intrinsic and extrinsic factors, and their essential role in muscle regeneration throughout the lifespan of an organism.

A stem cell is defined as a cell that can generate more stem cells indefinitely (self-renewal), while also producing cell progeny that mature into more specialized, tissue-specific cells (committed progenitors). When a stem cell self-renews, it divides to make copies of itself. The balance between populations of stem cells and differentiating cells is critical for embryonic development, and for long-term maintenance and regeneration of adult tissues. Unlike other tissues with high turnover such as blood, skin, and other epithelia, skeletal muscle in steady state conditions has a very slow turnover, with only ~1–2% of myonuclei replaced every week. This feature has made it difficult to characterize its regenerative potential. Mononucleate cells interspersed along the length of muscle myofibers, termed 'satellite cells', were identified nearly five decades ago using electron microscopy by Alexander Mauro. However definitive proof that this cell population includes functional adult stem cells essential for tissue regeneration has emerged only recently.

SATELLITE CELLS IDENTIFICATION

Skeletal muscles are composed of bundles of contractile muscle fibers (myofibers) that are large, terminally differentiated, multinucleate cells formed by the fusion of mature mononucleate progeny of MuSCs. The canonical MuSC in adult animals, designated "satellite cell," was anatomically defined in 1961 by transmission electron microscopy studies of the peripheral region of muscle myofibers in the *tibialis anticus* muscle of the frog. The discovery of the satellite cell heralded the birth of the field of muscle regeneration. Satellite cells have a high ratio of nucleus to cytoplasm, and are intimately juxtaposed to muscle myofibers. They reside in their own membrane-enclosed compartment, between the sarcolemma of the myofiber and the surrounding basal laminal membrane (Figure 30-1). Before the discovery of the satellite cell, it was unclear whether a mononucleate cell existed in mammals with the sole purpose of repairing damaged muscle myofibers, or whether the nuclei of damaged myofibers underwent a process of dedifferentiation, replicated as

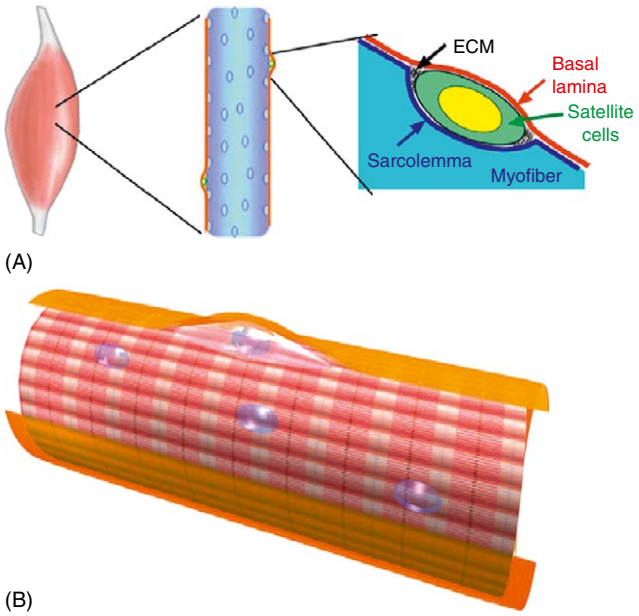


FIGURE 30-1 (A) Satellite cells are located between the sarcolemma of skeletal muscle fibers and the surrounding basal lamina. (B) 3D scheme of a muscle fiber with its satellite cell. Illustration by John Ramunas.

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mononucleated cells and then fused to form myofibers *de novo*, as in newts and axolotls.

Several lines of evidence supported the hypothesis that satellite cells possess MuSC function in mice. First, electron microscopy (EM) studies showed that after a single injection of [^{3}H] thymidine, only a small number of nuclei within satellite cells, but not nuclei of the myofiber, had incorporated the radioactive label, suggesting that satellite cells were quiescent most of the time, and were the only muscle-associated cells that proliferated. A subsequent study showed that after transplantation of a labeled [^{3}H] thymidine-extensor digitorum longus (EDL) muscle into the muscle bed of another animal, multiple myonuclei of the myofibers of the host animal were [^{3}H] thymidine labeled. The damage inflicted to the muscle bed during the transplantation procedure caused the satellite cells of the donor EDL to proliferate and differentiate, contributing donor myonuclei to the host myofibers. Taken together, these data suggest that satellite cells were stimulated to multiply and contribute to muscle in response to tissue damage. More recent studies analyzing BrdU incorporation confirmed these earlier results.

The second line of evidence that satellite cells possess MuSC activity derived from experiments that showed that, like other populations of proliferative cells, satellite cells are sensitive to γ-irradiation, which renders them unable to meet the demands imposed by muscle stress stimuli, such as increased weight or exercise. When part of the murine *tibialis anterior* (TA) muscle is surgically removed, the demands on the neighboring EDL muscle are increased, and because of the additional load the muscle must support, the EDL increases in mass as a result of a hypertrophic adaptive response. The gram weight of the EDL increases, as does the average myofiber diameter, compared with the EDL of the contralateral leg that did not have the TA resected. Notably, if one limb was treated with a high dose of γ-irradiation (25 Gy) before TA resection, the EDL could not adapt as well, and was both reduced in mass and had significantly smaller myofiber diameters than nonirradiated controls. These results provided evidence that the activity of satellite cells is linked to the hypertrophic response, an observation confirmed by others.

A third line of investigation that implicated satellite cells as the primary regenerative cell of skeletal muscle derived from the characterization of animal models with muscular dystrophy. One of the most commonly used mouse models of Duchenne muscular dystrophy (DMD) (among the most severe and common of the human heritable muscle wasting disorders) is the *mdx* mouse mutant, which carries a point mutation in the dystrophin gene that creates a translational block leading to a truncated protein. Dystrophin is a key protein in the membrane-bound dystrophin glycoprotein complex (DGC), which is thought to fortify the plasma membrane of the myofiber against the intense shearing forces that are generated during daily exercise. The muscles of the *mdx* mice have spontaneously revertant muscle myofibers that express dystrophin, due to a compensatory point mutation that corrects the translational block. As the mice age, revertant myofibers are found in clusters, in which each constituent myofiber harbors the same compensatory mutation. The compensatory mutations found in different bundles are unique to each bundle. This finding can be explained by experiments in which limiting dilutions of β-galactosidase (β-gal) encoding retroviruses were used to demonstrate that a satellite cell infected with a single retrovirus has the ability to proliferate and migrate laterally, participating in regeneration of not only its own myofiber, but also of nearby myofibers, leading to bundles of myofibers regenerated by the progeny of the same satellite cell. Taken together, these data suggest that the bundles of revertant myofibers in the *mdx* mouse are the result of fusion and differentiation of a clonally expanded satellite cell.

A fourth line of experimentation showed that myoblasts derive from MuSCs. MuSCs are typically cultured on collagen-coated or gelatin-coated plates from crude preparations of enzymatically digested skeletal muscle. When isolated by this method, they are usually termed myoblasts. These cells, if grown at clonal density, can differentiate into multinucleate myotubes when cultured in low-mitogen media, can fuse with existing myofibers when injected *in vivo*, and are considered to be the highly-proliferative descendants of satellite cells. However, because of the method by which they were isolated, their exact origin and their relationship to the anatomically defined satellite cell have been unclear. Recent evidence has shown definitively that myoblasts arise in culture from satellite cells contained within individual myofibers that are physically dissociated with great care so that they remain intact. That such single myofibers still harbored cells in the satellite cell position underneath the basal lamina was shown by scanning EM. With time in culture, mononucleated cells migrate off the myofibers, proliferating and forming colonies that differentiate into myotubes.

Fifth, striking evidence that satellite cells are essential for postnatal muscle growth and adult tissue regeneration comes from the generation of a mouse model nullizygous for the gene encoding the Pax7 transcription factor. Pax7 expression is characteristic of all satellite cells, and is lost once the cells differentiate. In mice, the knockout of Pax7 leads to an almost complete absence of satellite cells in muscles, and their postnatal growth and regeneration after injury is so severely impaired that they die within the first few months of life.

Finally, certain physiological and biochemical functions that correlate with satellite cell activity, or lack of activity, suggest that these cells are essential to muscle regeneration. One of the first physiological studies showing the dependence on MuSCs for an adaptive response in the EDL muscle was described earlier. In addition, in the fast myofibers of

the cat *plantaris* muscle, denervation and consequent immobilization causes muscle atrophy, leading both to a decrease in myonuclear number and fiber diameter compared with controls. Conversely, innervated *plantaris* muscle bearing increased weight exhibited augmentation of both myonuclear number and fiber diameter. These results are consistent with the interpretation that the satellite cells respond to exercise-induced muscle damage or stress.

Taken together, these findings demonstrate that the satellite cell is largely responsible for regeneration and maintenance of adult skeletal muscle, and strongly support the hypothesis that satellite cells can function as MuSCs, although this an hypothesis that was only recently confirmed definitively. To meet the classic definition of a tissue-specific stem cell required a demonstration that a single satellite cell transplanted into the muscle of a live mouse could yield more Pax7-expressing stem cells, and could contribute to mature multinucleated muscle myofibers. This was achieved by depleting the endogenous stem cell reservoir of recipient mice by irradiation of the TA muscles, and using genetically marked luciferase-expressing donor cells that could engraft if recipient mice were immunodeficient. Following enzymatic dissociation of muscles, enrichment by fluorescence-activated cell sorting (FACS) and injection as single cells into muscle, cells were monitored by bioluminescence imaging. This non-invasive method of monitoring cell numbers over time enabled identification of the mice which harbored substantial numbers of labeled cells that had to have derived from the single injected cell. Of a total of 144 mice injected with a single satellite cell, 4% exhibited a significant luciferase signal. In-depth analysis showed that in these mice, prospectively isolated MuSCs fulfilled the classical definition of a tissue-specific stem cell: the dual capacity for self-renewal and the generation of more specialized progeny.

SATELLITE CELL HETEROGENEITY

For a long time, muscle cells were isolated from tissues by enzymatic digestion followed by cell culture that would select for *myoblasts*, a population capable of dividing *in vitro*. However, it should be stressed here that cell populations obtained following growth in culture suffer from the following limitations:

1. They are likely to represent a subset of the total cells, as the cells that grow will have resulted from *in vitro* selection. To clarify, cells with the ability to propagate in culture will start dividing and overtake nondividing cells, resulting over time in a population of cells with very different features from those initially isolated. As a result, the cells may well not reflect the *in vivo* population from which they derived.
2. Long-term culture could result in the accumulation of DNA mutations that may confer a proliferative advantage, again leading to a shift in the population under study. For these reasons, although a useful tool for certain studies as they are easy to obtain in quantity, study and manipulate, cells established in culture should not be considered representative of stem cells in the intact animal. A recent study exemplifies the differences between freshly-isolated and cultured muscle stem cells. Whereas the freshly-isolated cells extensively contributed to tissue regeneration in a model of muscular dystrophy, satellite cells kept in tissue culture for four days lost their regenerative potential, and exhibited a very limited ability to repopulate and restore the muscle of dystrophic mouse recipients.

As a result, major efforts by a number of researchers have been devoted to study of satellite cells immediately after isolation, and to evaluate their function after transplantation into recipient mouse muscles with no intervening culture step. One approach involved developing strategies to isolate and transplant single genetically marked myofibers with their associated satellite cells into recipient mouse muscles. A second approach involved developing protocols for prospectively isolating satellite cells, based on expression of transgenes or proteins they express on their surface, using antibodies coupled to fluorophores that enable separation of the cells by FACS. These approaches obviate the culture step, and allow an analysis of the stem cells immediately upon transplantation into adult muscles of mouse recipients. Such studies have provided several lines of evidence that cells in the satellite cell position are heterogeneous, comprised of both stem cells and more committed progenitors, based on their patterns of gene expression.

First, by taking advantage of the ability to isolate and culture single myofibers, satellite cells were analyzed for expression of key markers, such as the transcription factors, Pax7 and MyoD, by immunostaining. These studies showed that the satellite cell population differentially expressed these two markers, suggesting that different populations with different functions or hierarchical relationships may coexist within this anatomical compartment. Second, exacting studies have shown that when a single genetically-marked myofiber with its satellite cells is isolated from one adult muscle subtype, and transplanted into another adult muscle subtype, it is capable of extensive contribution to both the host satellite cell population and to the regeneration of the mature tissue (myofibers) after serial tissue injury. These studies also revealed that myofibers derived from different muscles (e.g., fast and slow contracting) exhibited pronounced differences in their ability to regenerate recipient muscles, indicating that the satellite cells in the adult animal are heterogeneous. Moreover, if single myofibers are exposed to a snake venom toxin, notexin, which damages muscle myofibers, just prior to transplantation, their regenerative potential remains unaffected,

demonstrating that satellite cells can exert their function *in vivo*, even when they are artificially removed from their physiological niche (their own myofiber).

A third line of evidence in support of satellite cell heterogeneity was provided by studies that took advantage of a lineage-tracing strategy by using the Cre-lox system. In these studies transgenic mice which express the Cre recombinase protein under the control of the Myf5 promoter (a transcription factor characteristic of satellite cells) are crossed with Cre reporter mice, in which the YFP (yellow fluorescent protein) is expressed only if a Cre-mediated recombination event takes place in the genomic DNA. The progeny of these mice have YFP+ satellite cells (~90%, in which the promoter of the Myf5 gene, which is typically expressed in satellite cells, was active at some point during development) and YFP− cells (~10%), in which Myf5 activity was never present at sufficient levels to induce a recombination event. A characterization of these two distinct subpopulations has shown that the YFP− cells possess a much higher regenerative potential after transplantation, and can give rise to both more YFP− cells and more YFP+ cells. These results further substantiate that there is heterogeneity among the cells that occupy the adult satellite cell compartment, highlighting the need for a rigorous characterization of MuSCs at the clonal level.

Fourth, single MuSC gene expression studies have also revealed heterogeneity. In these experiments, satellite cells were prospectively isolated and enriched by FACS using fluorescent antibodies to α7-integrin and CD34 cell surface markers, characteristic of satellite cells. RT-PCR was performed simultaneously for four different transcripts characteristic of either satellite cells (Pax7 and Myf5), or more specialized progenitors (Pax3 and MyoD). Although 100% of the cells expressed mRNAs encoding the satellite cell-specific transcription factors, a subset also expressed the progenitor-specific transcripts. These results demonstrate that the satellite cells are not all at the same stage of commitment. Further evidence is provided by dilution analyses. When 10 FACS-enriched MuSCs are injected into an irradiated recipient muscle, engraftment in 16% of mice is observed, indicative of stem cell function. When only one cell is injected, ~4% of isolated adult MuSCs display the ability to self-renew and differentiate *in vivo*, i.e., to generate progeny on division that include more stem cells that stably persist within the host muscle and also give rise to committed progenitors that contribute to muscle regeneration. Although this frequency may overestimate the heterogeneity of the population, as the procedure for single cell isolation and injection could lead to substantial death, the results support the general finding that satellite cells are not all the same.

Altogether, these reports demonstrate that the satellite cell compartment is heterogeneous, comprised of both stem cells and progenitors. Whether the progenitors are in a transient state between stem cells and differentiation, or whether they persist long-term and serve a different function from the satellite cells (i.e., responding to different microenvironmental stimuli), still remains to be established. Additional methods and markers for further distinguishing sub-populations of satellite cells will be useful in distinguishing between these possibilities. To date, skeletal muscle researchers have employed FACS using combinations of diverse cell surface markers to prospectively isolate satellite cells such as CD34, α7-integrin, β1-integrin, CXCR4, Syndecan4, or used reporter genes such as myf5-YFP or Pax3-GFP. Moreover, other sources of cells have been identified that can efficiently contribute to muscle regeneration, such as mesoangioblasts and muscle-derived stem cells (MDSC). These cells are not prospectively isolated, but instead are selected over time using cell culture regimens. Although these cell populations may well have therapeutic potential, their role in normal development and function as self-renewing stem cells *in vivo* has not been ascertained, and currently they do not rigorously fit the stem cell definition. A direct comparison of these cell populations could now be performed using *in vivo* non-invasive imaging assays in conjunction with histological assays.

SATELLITE CELL SELF-RENEWAL

Self-renewal is a necessary process in adult life to maintain the tissue-specific stem cell pool. Several mechanisms have been described to maintain and expand the number of stem cells in a tissue. In the case of symmetric division, a stem cell divides symmetrically giving rise to two daughter stem cells, thus expanding the stem cell pool. In the case of asymmetric division, a stem cell divides to give rise to one daughter stem cell and one daughter committed progenitor, which will soon differentiate, thus maintaining the stem cell pool. Finally, reversion could result if a committed progenitor stops dividing and goes back to a quiescent stem cell state, thereby filling a vacant niche. In skeletal muscle, several studies suggest the occurrence not only of symmetric, but also asymmetric divisions in satellite cells, a mechanism already described in other stem cell types. Asymmetric division maintains homeostasis and several molecular mechanisms have been implicated for adult muscle satellite cells.

DNA Segregation

Pulse-chase labeling experiments have been used for this purpose which employ halogenated thymidine analogs (bromodoxyuridine, BrdU, chlorodeoxyuridine, CldU, and iododeoxyuridine, IdU), which incorporate into the genome on DNA synthesis during the S-phase of the cell cycle, and can be detected by specific antibodies. Pioneering studies were performed on intestinal and neuronal stem cells and embryonic fibroblasts that suggested that this is a way by which two daughter cells may adopt divergent

fates, with only one of them retaining the features, the "epigenetic signature" of their mother. Recent experiments in two laboratories revealed the occurrence at low frequency of asymmetric segregation of DNA on division of satellite cells both *in vitro* and *in vivo*, with the older templates (non-labeled with the analogs) co-segregating to one daughter cell and the younger templates (newly synthesized, labeled with the analogs) to the other. A characterization of the progeny indicated that the daughter cell inheriting the older templates retains a stem cell-like phenotype, while the one receiving the young templates has a more differentiated phenotype. These results provide support for the immortal DNA strand hypothesis in muscle, which was proposed many decades ago as a mechanism by which stem cells protect their genome from replication errors on division.

Protein Segregation

Another mechanism of self-renewal entails the asymmetric segregation of proteins (cell fate determinants) among daughter cells. Originally described in the central nervous system in *Drosophila* and mammals, segregation of proteins is a widely-employed mechanism by which two daughter cells assume divergent fates. In satellite cells *in vitro* it has been shown that on division, one daughter cell inherits the Notch inhibitor Numb, while the other does not. However, it is still controversial whether Numb segregation marks a daughter stem cell, or a more committed progenitor.

Mitotic Spindle Orientation

Recently it has been shown on isolated myofibers that a satellite cell can undergo division with two different orientations with respect to the myofiber surface: (1) planar, in which the mitotic spindle is parallel to the myofiber surface, generating two identical daughter cells (either both Pax7+Myf5− or both Pax7+Myf5+), both still in contact with the basal lamina and the plasma membrane of the myofiber; and (2) apical-basal, in which the spindle is perpendicular to the myofiber surface, generating a cell still in contact with the basal lamina (Pax7+Myf5−, cell retaining stem-like properties) and a cell which loses this contact and remains close to the myofiber membrane (Pax7+Myf5+, more committed progenitor). Stem cell polarity and spindle orientation have been shown to play a central role in stem cell asymmetric division in several organ systems including muscle, underscoring the instructive role of the microenvironment, or niche, in determining stem cell fate.

Reversion

Reversion from a committed state to a stem cell-like state is an intriguing mechanism first demonstrated to occur in *Drosophila* testis. Whether reversion serves as a mechanism for generating stem cells in mammals remains to be established. Some recent experiments suggest that this may be the case for murine muscle. When single myofibers are cultured in suspension, satellite cells maintain their sublaminar position, which allows their clonal analysis when they start dividing. In these experimental conditions, quiescent satellite cells undergo activation, evident as co-expression of Pax7 and MyoD. Later, although the majority of the progeny lose Pax7, and soon differentiate and express MyoD, some cells appear to lose MyoD. Indeed, it is possible to observe within the same cluster some cells that are Pax7+MyoD−, suggesting that an activated Pax7+MyoD+ satellite cell gave rise to a cell that later lost MyoD, retained Pax7, and resumed a quiescent, stem-like state.

INTRINSIC REGULATION OF SATELLITE CELLS

Several genes have been identified as key regulators of muscle satellite cell specification and activation, including transcription factors and proteins involved in pathways transducing signals from the microenvironment. The largest body of evidence comes from studies of knockout mouse models, in which one or more genes have been ablated by genetic manipulation.

Pax Genes

The Pax7 gene is a member of the paired-box containing gene family, and it is selectively expressed in quiescent and activated satellite cells, but not in differentiated cells. Mice nullizygotes for Pax7 display an almost total absence of satellite cells and their muscles are severely impaired in postnatal growth and tissue regeneration, underscoring the central role of Pax7 in satellite cell specification. Pax3 was shown to have a role in skeletal muscle when a mutation in the gene was identified as the basis for the Splotch mouse mutant, a spontaneous mutant originally described in 1947. This mutant is not viable, it survives until day 15 of gestation, and displays defects in the neural tube, heart, and a striking absence of limb musculature, due to a defect in migration of myogenic progenitors during development. Pax3 was later also shown to be essential for the determination of myogenic progenitors in the somites during early development.

During development, Pax3 and Pax7 mark a population of myogenic progenitors that will give rise to the satellite cells of the postnatal muscle. Interestingly, while in adult resting muscle Pax7 marks all quiescent and activated satellite cells, Pax3 expression persists only in few muscle masses, including the diaphragm, but not the limbs. Recently, it was shown that during satellite cell activation in the limb, Pax3 is transiently up-regulated, and then

down-regulated post-translationally through monoubiquitination, which targets the protein for proteasomal degradation. The degradation of Pax3 in the late phases of satellite cell activation is essential for terminal differentiation. Thus, Pax3 in limb muscles marks transient amplifying progenitors, an intermediate stage between satellite cells and myoblasts. The basis for this heterogeneity among satellite cells derived from different adult muscles is still poorly-understood. Indeed, when Pax3-expressing satellite cells are transplanted into the muscles of the limb, they appear capable of proliferating and contributing to muscle regeneration. Since Pax3 is a marker of activated progenitors in the limbs, expression of Pax3 in the diaphragm could be indicative of a higher frequency of activated satellite cells in this muscle which, by contrast with limb, is typified by constant contractile activity necessary for breathing. In addition, recent studies have highlighted an anti-apoptotic role of both Pax7 and Pax3 in muscle stem cells, both in the embryo and in adult animals.

Pax7 and Pax3 are transcription factors; however, their target genes in adult muscles are only starting to be elucidated. For example, it has been recently reported that Pax3 activates the Myf5 gene, essential for myogenic progression, and that Pax7 activates the Myf5 gene by binding the Wdr5-Ash2L-MLL2 histone methyltransferase complex, that methylates histone 3 lysine 4 (H3K4) on the Myf5 gene, thereby promoting its transcription.

MRF Genes

During development, a key family of transcription factors essential for satellite cell specification and regulation is the basic helix–loop–helix transcription factor family, also known as Muscle Regulatory Factors (MRF), comprised of Myf5, MyoD, myogenin and MRF4. *In vitro*, proliferating primary myoblasts express both Myf-5 and MyoD, and then their expression declines during differentiation and is followed by the expression of myogenin and MRF4. Surprisingly, knockout mice for MyoD, once considered the master regulator of myogenesis, have normal muscles upon birth, but display a regeneration defect, likely due to the impairment of MyoD−/− satellite cells to efficiently differentiate to form myotubes. Knockout mice for Myf5 have rib but no evident muscle abnormalities, suggesting that other MRFs may compensate for Myf5 absence. Double MyoD/Myf5 mutant mice lack muscles, due to the absence of embryonic muscle progenitors, underscoring the role of these two gene products as myogenic determination factors. Notably, knockout mice for myogenin have the most pronounced phenotype, and die perinatally due to a defect in muscle differentiation, as shown by the absence of myofibers in these mice.

MRFs also play a role in adult life. A large proportion of satellite cells express Myf5. Upon muscle injury or degeneration, satellite cells undergo activation, a process which entails the activation of specific genes and re-entry into the cell cycle from their quiescent state. Upon activation, MyoD and Myf5 genes, two members of the MRF family, are up-regulated, and their expression persists throughout muscle regeneration until the tissue is repaired and homeostasis is reached. Furthermore, microarray gene profiling of MyoD−/− myoblasts revealed the up-regulation of several anti-apoptotic genes and overexpression of MyoD in MyoD−/− myoblasts induces cell death, identifying a putative novel role of MyoD in apoptosis. Together with the anti-apoptotic function of Pax7 and Pax3, these results suggest a balance between the anti-apoptotic role of Pax genes and the pro-apoptotic role of MyoD, indicating that muscle progenitors that lose Pax gene expression may be more prone to cell death during regeneration. These results are consistent with a recent study in which the dynamic behavior of Luciferase+ transplanted satellite cells was monitored by bioluminescence imaging, and it was possible to observe that, in response to tissue injury, donor derived cells underwent a massive proliferative response. Following this regenerative wave of cell expansion, substantial death of a proportion of donor-derived cells was observed, suggesting that cell death may be a physiological mechanism for reestablishing homeostasis after muscle tissue regeneration.

PW1 Gene

In an effort to identify muscle stem cell regulators, a differential screening approach identified PW1, a gene that encodes a large protein containing 12 zinc fingers and multiple proline-rich repeat motifs. PW1 is expressed in the early embryo and later in skeletal muscle, with high levels at a perinatal stage that decline progressively in adult life. *In vitro*, PW1 is expressed in primary myoblasts, and it has been shown to mediate the block of muscle cell differentiation induced by TNFα. Interestingly, PW1 expression is negligible in MyoD−/− primary myoblasts, which perform poorly in regenerating muscle. Recently, PW1−/− knockout mice were generated and display severe impairment in postnatal muscle growth. Satellite cells are present, but they fail to remain in a quiescent state. In adulthood, PW1 is found in satellite cells, and also in a subset of interstitial cells and vascular cells. Together, these results indicate that PW1 may be a key regulator of muscle stem cells. Further characterization of PWI-expressing stem cell populations will reveal their relative contribution to tissue homeostasis and regeneration, and establish the hierarchical relationship of PWI to other myogenic regulators.

EXTRINSIC REGULATION OF SATELLITE CELLS

The concept of a stem cell niche was first proposed by Schofield in 1978 as a dynamic entity, or microenvironment,

that regulates the balance between maintaining the resident stem cell in a quiescent state during homeostasis, as well as permitting self-renewal in response to activating factors. First described in *Drosophila melanogaster* and *C. elegans* ovary and testis, niches have now been characterized in mammalian bone marrow, skin, intestine, brain, and testis, as well as skeletal muscle. The identification of satellite cells by their anatomical location as mononucleated cells at the surface of the myofiber and ensheathed by the basal lamina demonstrates the importance of the niche to satellite cell identity. If physically removed from the niche and plated *in vitro*, satellite cells immediately begin to specialize as myoblasts and their potential for self-renewal and contribution to myofibers in muscle tissue diminishes rapidly, highlighting the significance of the niche to maintaining satellite cell function.

Satellite Cell Niche

The adult satellite cell niche is defined as the cells, protein factors, and overall architecture of the environment directly in contact with a resident satellite cell. The niche is comprised of a unique combination of biochemical features (cells with their receptors and secreted factors, matrix proteins, growth factors, cytokines) and biophysical features (mechanical and structural), cues capable of regulating satellite cell behavior. The satellite cell niche is currently defined by the presence of a satellite cell and, as such, the satellite cell serves as an integral part of the niche. This view could be challenged by the identification of components that expressly define the satellite cell niche, and that are identifiable even in the absence of a satellite cell. Regardless, in order for a satellite cell to home to, interact with, and be maintained within a niche, it must express certain receptors, the expression of which is either intrinsic to the satellite cell or induced upon exposure to the niche. It has been shown in several adult tissues that stem cell niches display an asymmetric distribution of components. Myofibers are known to express SDF-1, a migration signal that interacts with the receptor, CXCR4, which is expressed on satellite cells, as well as M-cadherin, a cell adhesion molecule. The myofiber is thought to exert a quiescent effect on satellite cells, either through its physical interaction with the myofiber or via signals secreted by the myofiber. In experiments in which the myofiber was selectively eliminated, leaving the basal lamina intact, resident satellite cells were induced to proliferate. Although the myofiber can regulate satellite cell behavior, the receptors and secreted signals expressed by the fiber have yet to be elucidated. The basal lamina is a proteinaceous matrix encapsulating the surface of the satellite cell, not in contact with the myofiber. It is composed primarily of type 4 collagen, laminin, fibronectin, and entactin, as well as glycoproteins and proteoglycans, that interact with one another to form a structural network. In addition to providing a substrate to which satellite cells can anchor, the basal lamina provides a physical barrier between the satellite cell and neighboring myofibers. Also, receptor ligand components of the basal lamina, such as laminin and fibronectin, can regulate satellite cell behaviors, such as quiescence and proliferation, while other components, like proteoglycans and perlecan, can function as receptors for factors that under steady state conditions inhibit growth, yet can mediate growth when activated as a result of injury and act to prime the niche for a satellite cell response.

Satellite Cell Microenvironment

Satellite cell function can also be influenced by factors in the environment beyond the immediate satellite cell niche. Within the connective tissue resides a population of cells, whose function has yet to be elucidated. In addition, there are fibroblasts which deposit large amounts of extracellular matrix components and secrete growth factors, both of which can alter satellite cell function. Recent studies suggest that satellite cells are preferentially localized close to the microvasculature within the muscle. The notion of a vascular niche has also been proposed for neuronal and hematopoietic stem cell niches in the CNS and bone marrow, respectively. Notably, VEGF and other growth factors and enzymes secreted by endothelial cells can activate satellite cells to proliferate and expand. Satellite cells have also been shown to preferentially cluster near neuromuscular junctions. It is unclear whether components of the neuromuscular junction influence the resting satellite cell, but on denervation, satellite cells are activated to proliferate, likely due to a combination of myofiber disruption and neurotrophic factors. Finally, infiltrating immune cells recruited for repair, as well as circulating systemic factors, can both influence satellite cell function.

Satellite Cell Niche Heterogeneity and Occupancy

The satellite cell population within adult skeletal muscle is functionally heterogeneous. For example, as adult skeletal muscle ages, a large proportion of satellite cells are lost, but there is a small population that persists and is able to repair damage. Similarly, upon exposure to high doses of irradiation, the majority of satellite cells are ablated, but there is a functional, radiation-resistant population that remains. It is thought that differential expression of intrinsic factors promotes heterogeneity within the population, but extrinsic factors may also contribute to satellite cell heterogeneity. Niche heterogeneity could arise simply because some niches are “spared” during insult, and these spared niches harbor functional satellite cells. However, another intriguing possibility is that niches are inherently heterogeneous in terms of their resistance to specific types of insult, leading to sub-populations of satellite cells with

the capacity to survive and function in specific situations. If there were an infinite number of satellite cell niches to fill, one might expect to find many satellite cells per fiber, and little consistency in terms of the number of satellite cells per fiber. On the contrary, skeletal muscle myofibers have relatively few satellite cells per fiber, and myofibers within a muscle type have a relatively constant number of resident satellite cells. Indeed, recent studies demonstrated that prospectively isolated satellite cells transplanted into irradiated hindlimbs undergo an initial period of proliferation, but eventually cell growth plateaus. In addition, in response to sequential notexin damage, donor derived cells undergo a second round of proliferation that exceeds the need of the tissue, resulting in a decline in cell numbers until the tissue again reaches homeostasis. The observation that skeletal muscle adapts to achieve homeostasis after radiation or toxin injury suggests that the tissue has feedback mechanisms that operate to maintain a constant number of satellite cells within their niches. How skeletal muscle myofibers maintain a specific number of niches might be best studied by comparing the development of soleus muscle, which has a relatively large number of satellite cells per fiber (20 satellite cells/fiber) to a muscle with fewer satellite cells per fiber, such as the tibialis anterior (10 satellite cells/fiber).

The Aging Satellite Cell Niche

The composition and relevance of the satellite cell niche is most readily revealed during skeletal muscle aging, injury, and disease. During the process of aging, the overall number and functionality of adult satellite cells appears to decline. Of particular interest are several lines of evidence suggesting that these remaining cells have the intrinsic ability to repair injury, but are inhibited by changes incurred to the niche. First, satellite cells isolated from old mice and transplanted into young recipients are able to engraft and repair damaged muscle, demonstrating that the aged donor satellite cell maintains its intrinsic regenerative potential, and is inhibited by the aged environment. Moreover, heterochronic parabiotic pairings of young and old mice (mice that were surgically joined so that they share the same circulation) showed that satellite cells within old muscle can be rejuvenated by factors within the systemic environment of the attached young mouse. Conversely, when young satellite cells are transplanted into old hosts they exhibit a marked decline in regenerative capacity. Together, these studies demonstrate that the satellite cell niche exerts a critical control over satellite cell function, and that when it incurs changes, such as during aging, this can significantly impede normal skeletal muscle repair. Recently, activation of Notch signaling, which promotes satellite cell proliferation and production of myoblasts necessary for repair, was shown to be lost with age, correlating with a loss of regenerative potential. These studies revealed that while satellite cells within the aged muscle expressed Notch receptors, the Notch ligand, Delta, was no longer being supplied by cells in the microenvironment. Consistent with this finding, enforced Notch signaling was able to restore the regenerative potential of aged muscle. In additional studies, it was demonstrated that Wnt3a is up-regulated during aging and can convert myogenic precursors to cells of fibrogenic lineage, thereby promoting fibrosis. These observations pose the interesting possibility that the niche itself could be targeted therapeutically, by supplementing factors lost during aging or disease, and underlies the importance of characterizing the composition and function of normal, aging, injured, and diseased satellite cell niches.

Biophysical Properties of the Satellite Cell Microenvironment

In addition to biochemical factors, several lines of evidence demonstrate that cells can both sense and respond to biophysical cues. For example, mesenchymal stem cells cultured on a polyacrylamide substrate engineered to mimic the stiffness of brain, muscle, or bone lead to distinct cell morphologies and the expression of markers consistent with differentiation into brain, muscle, or bone, respectively. In addition, cultured myoblasts can be induced to differentiate into myotubes and exhibit muscle striations, simply by culturing them on polyacrylamide gels with mechanical properties similar to endogenous muscle. Although it is important to note that all of these studies were performed *in vitro*, they clearly demonstrate the capacity of cells to respond to mechanical cues in a directed manner. Whether or not mechanical properties can influence the function of satellite cells has yet to be determined. However, it is known that the mechanical properties of muscle are altered during aging, injury, and disease, and that these changes are likely due to increased fibrosis from the influx of collagen-producing fibroblasts observed during chronic regeneration. In addition to mechanical properties, the structure and three-dimensional architecture of the satellite cell niche likely plays a role in regulating satellite cell fate. The current increase in integrative science between engineers and biologists will advance these concepts significantly in the coming years, with the advent of novel technologies to study the relevance of biophysical properties on stem cell fate both *in vitro* and *in vivo*.

The satellite cell niche is a protective and instructive environment that is the target of aberration during skeletal muscle aging, injury, and disease. Research aimed at elucidating the biochemical and biophysical composition of the resting and damaged niche, and how that composition impacts satellite cell function, could aid in the development of novel “niche”-targeted therapies.

SUMMARY

In the last 10 years significant progress has been made in understanding adult muscle stem cell (MuSC) identity and function. The demonstration that MuSCs can be transplanted, even when removed from their physiological niche (muscle fiber), and still maintain their function, opened the door to a new phase of studies in which MuSC could be prospectively isolated using a combination of surface markers and FACS. The function of these cells could then be ascertained by transplantation into recipient animals. Such studies allowed a clear demonstration that the satellite cell compartment is comprised of cells that are heterogeneous, including both stem and progenitor cells. Furthermore, the use of novel strategies to monitor stem cell behavior *in vivo*, such as non-invasive bioluminescence imaging, provided insights into the dynamics and kinetics of MuSC proliferation *in vivo* that will be extremely useful in the future in understanding stem cell function and contribution in a quantitative manner in models of muscular dystrophy. In parallel, elegant studies have recently highlighted the instructive role of the niche in directing MuSC behavior *in vivo*, and the molecular mechanisms involved are now starting to be elucidated.

The current challenge is to isolate and characterize human MuSCs, and to develop strategies to promote their expansion *in vitro*, for the development of cell-based therapies to counteract tissue degeneration in muscle wasting diseases, such as muscular dystrophy. In addition, the discovery of drugs capable of stimulating resident satellite cells could counteract muscle loss with aging.

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KEY WORDS

MuSC *Mu*scle *s*tem *c*ells; mononucleate cells that function to regenerate damaged myofibers through a process of proliferation, followed by subsequent fusion with existing or nascent myofibers.

Myofiber Large multinucleate cells that contain a contractile apparatus, composed primarily of actin and myosin family proteins, to facilitate movement. Muscle groups are composed bundles of myofibers.

Satellite cell An anatomical designation of a mononuclear cell that sits on a myofiber juxtaposed to the plasma membrane of the myofiber, yet is ensheathed by the basal laminal membrane, which also surrounds the myofiber.

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Chapter 31

Stem Cells and the Regenerating Heart

Maria Paola Santini, Bhawana Poudel, and Nadia Rosenthal

INTRODUCTION

In contrast to mammalian skeletal muscle, which regenerates injured tissue through activation of quiescent myogenic precursor or multipotent adult stem cell populations, cardiac muscle does not appear to retain equivalent reserve cell populations to promote myofiber repair. Interruption of the coronary blood supply results in apoptosis and fibrotic scar formation, at the cost of functional muscle. As a result, the remaining cardiomyocytes undergo cellular hypertrophy, leading to decompensated function and congestive heart failure, an increasingly prevalent disease in the industrialized world.

The impediment to adult mammalian cardiac regeneration has been attributed to its distinct embryonic history, and to the relative paucity of progenitor cells residing within the heart. The heart is the first fully-differentiated structure to form and function during vertebrate development. The primitive heart tube, composed of contracting cardiomyocytes lined by a layer of endocardial cells, ensures the establishment of a circulatory system which is critical to support rapid rates of embryonic growth. Actively contracting fetal cardiomyocytes must continue to divide to provide for further growth of the embryonic heart. This phase of cardiomyocyte division ends soon after birth, when increases in myocardial mass are achieved largely through cellular hypertrophy, at which point the heart is considered fully-developed.

Lineage diversification in the embryonic heart arises via distinct pools of cardiovascular progenitors that are segregated early in the developing embryos to form the four specialized chambers, the coronary arterial tree, the outflow tract, the endocardium and the heart valves (Laugwitz *et al.*, 2008). Of these cardiac progenitor cells, only those positive for the transcription factor Isl1 have been traced and analyzed *in vitro* (Laugwitz *et al.*, 2008). These cells take part in the formation of the secondary heart field and decrease notably after birth, rendering a prominent role in regeneration in the working adult heart unlikely (Laugwitz *et al.*, 2008). Although adult cardiomyocytes have been shown to proliferate during both pathological and physiological conditions in the adult heart, division events occur at a low rate to counteract the devastating effects of myocardial infarct. Additional distinctive factors to provide a genetic fingerprint of precursor cardiac cells in the adult heart have yet to be discovered, so that isolation and therapeutic use of cardiac progenitor cell (CSC) populations remains elusive.

In the last years, strategies have been proposed to regenerate the heart via cell therapy, combined with tempering the hostile environment of the infarct, by administration of cell survival and anti-inflammatory molecules. Important advances in the control of stem cell fate have also moved the field of regenerative medicine closer towards applicable therapies for cardiac muscle regeneration. However, the field has yet to overcome significant obstacles, including the incomplete cell differentiation of stem cells (with the associated danger of tumor formation), the paucity of organ-specific stem cell resources (specifically in adult/aged organs), and the immunogenicity of the transplanted cells (graft rejection).

The Elusive Cardiac Stem Cell

The adult mammalian heart has traditionally been considered a post-mitotic organ without an endogenous population of stem cells, and with a relatively constant number of myocytes that cease to divide shortly after birth and remain constant into senescence. Reversal of cardiac damage implies generation of new contractile myocytes, and formation of a capillary network able to support the greater demands of the regenerating myocardium. A long-standing view is that the inability of differentiated cardiac myocytes to re-enter the cell cycle may present the ultimate impediment to the heart's regenerative capacity, and be responsible for the drastic effects of acute and chronic myocyte death in the surviving myocardium after infarction. Athough most of the controversy surrounding mammalian heart regeneration has focused on the evidence for or against the replication

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of existing myocytes, cells capable of differentiating into a myocyte in the adult heart could originate through the commitment of precursor cells to the myocyte lineage, through replication of pre-existing myocytes, or by a combination of these two mechanisms.

The origin of cycling myocardial cells has been attributed to Lin−/c-kit+ cells isolated from the adult rat heart that retain stem cell characteristics. These cells are self-renewing, clonogenic, multipotent *in vitro* and *in vivo*, and give rise to myocytes, smooth muscle and endothelial vascular cells (Schuldt *et al.*, 2008). When injected into an ischemic rat heart, a population of these cells, or their clonal progeny, reconstitutes large portions of the injured myocardial wall (Schuldt *et al.*, 2008). The regenerated myocardium contains small myocytes that present the anatomical, biochemical, and functional properties of young myocytes. Recent evidence shows that Lin−/c-kit+ cells also have the ability to form conductive and intermediate-sized coronary arteries when injected in infarcted rat heart (Schuldt *et al.*, 2008). Resistance arterioles and capillaries are connected with primary coronary circulation, indicating that Lin−/c-kit+ cells have a dual role in vessel and contractile muscle formation. Of note is the discovery of three additional populations of resident cardiac progenitor cells (CPCs) identified by the stem cell antigen-1 (Sca-1), Hoechst exclusion (side population, SP), and the presence of the transcription factor isl1. Among them, the Lin− c-kit+ and Sca-1+ population have shown evidence of mature sarcomere formation *in vivo*, whereas isl1 positive cells generate action potentials comparable to those of neonatal myocytes *in vitro*, although their regenerative properties have not been tested *in vivo* (Schuldt *et al.*, 2008).

Taken together, these data support the notion that myocyte renewal in the adult mammalian heart occurs constantly, albeit at a very low rate (a single Lin− c-kit+ cell is present every 10^4 myocytes, and only 0.03% of total heart cells are SP cells). The therapeutic potential may therefore be limited by the difficulty of obtaining a sufficient number of cells from a patient's biopsy. A method of rapidly expanding a population of CSCs from heart biopsies, which grow into spherical structures termed cardiospheres, shows some promise: cardiosphere-derived cells improved left ventricular function after MI, and showed spontaneous action potentials *in vitro* when co-cultured with neonatal rat ventricular myocytes (Messina *et al.*, 2004). Although these studies generally support the notion that CSCs meet *in vitro* and *in vivo* criteria for differentiation into cardiac myocytes and endothelial cells, no consensus has yet been reached on the identity and characteristics of individual CSC types.

Recruiting Circulating Stem Cell Reserves

The presence of a circulating cell population that could restore the heart has gained credibility from observations of sex mismatched cardiac human transplants in which a female heart is transplanted into a male host. In these patients, the presence of Y-chromosome-positive myocytes and coronary vessels in the transplanted female hearts proves the existence of migratory host-derived precursor cells that are induced to differentiate by the cardiac milieu (Schuldt *et al.*, 2008). Although this phenomenon could be a response to organ transplantation, it may reflect a normal homeostatic process for the maintenance of cardiac muscle and coronary vasculature. Indeed, a recent report showed that the heart possesses regenerating capacities in which stem or precursor cells "refresh" adult mammalian cardiomyocytes after ischemia or pressure overload, but not during aging (Dimmeler *et al.*, 2008).

The dearth of information regarding the precise origin of cardiac precursor cells has prompted animal experiments in which stem cells isolated from bone marrow were enriched for various surface markers and analyzed for their regenerative properties to restore cardiac function. The initial controversy over the capacity of c-kit positive bone marrow cells (BMCs) to form cardiomyocytes and synchronously contract with the other cardiomyocytes still remains open. In a murine infarct model delivery of Lin−/c-kit positive BMCs carrying the enhanced green fluorescent protein (eGFP) promoted repopulation of the infarcted heart with new myocytes (Schuldt *et al.*, 2008). Other groups have not been able to reproduce this initial result, and additional investigations have indicated that BMCs preferentially fuse to existing cardiomyocytes, explaining the appearance of myocytes labeled with eGFP (Dimmeler *et al.*, 2008).

Despite the lack of agreement in the outcomes of these reports, the majority of studies in models of peripheral ischemia showed that BMC infusion in infarcted murine hearts led to improvement of global cardiac function and enhancement of blood flow. Based on these results, human clinical trials were initiated in 2001 to treat patients with cardiac ischemia with autologous circulating blood or bone marrow-derived cells. The initial pilot studies (TOPCARE-AMI, BOOST trials) showed an improvement in global left ventricular ejection fraction from 6% to 9% (Dimmeler *et al.*, 2008; Schuldt *et al.*, 2008). These results were confirmed in a larger, double-blind, randomized trial called REPAIR-AMI. In this study the global and regional ejection fraction in the bone marrow-treated group improved modestly but significantly, by 2.9% compared to the placebo-treated group. Notably, a larger study called ASTAMI trial did not show any benefit on the left ventricular functional parameters (Schuldt *et al.*, 2008). Although the two trials differed in the treatment of the isolated cells that might affect their functional capacity, long-term studies showed that the beneficial effect of injected BMCs observed after six months were no longer present at 18 months, suggesting that the main effect was an acceleration of recovery (Schuldt *et al.*, 2008).

Taken together, the data to date suggest that functional benefits may be mediated through paracrine secretion of growth factors or cytokines more than through transdifferentiation of BMCs into cardiomyocytes. Secreted factors could indirectly promote survival of cardiomyocytes, mobilization of endogenous progenitor cells, or neovascularization. Unfortunately, convincing evidence from multiple laboratories has yet to emerge, and differences in experimental design have generated confusion about the relative benefits of transplanted bone marrow cells in treating cardiac ischemia.

It is, nevertheless, likely that myocardial recovery after infarct can be elicited by modulating the intrinsic resources of the heart. One of the impediments to effective cardiac regeneration, at least in mammals, may be the relatively poor recruitment of circulating stem cells to the site of myocardial injury limits the body's ability to aid in the repair process. Numerous chemotactic signals associated with inflammation, including cytokines and adhesion molecules, are preferentially expressed by the infarct border zone, and might be augmented to favour stem cell homing. Indeed, increasing evidence supports the notion that chemokines play a central role in directing cells from the bone marrow to ischemic myocardium. Transplantation of cardiac fibroblasts engineered to chronically express SDF1 into the myocardium of animals two months after myocardial infarction resulted in the recruitment of bone-marrow-derived hematopoietic stem cells, vasculogenesis, and improved cardiac function, independent of regeneration of cardiac myocytes (Penn, 2007). In rats, the re-expression of monocyte chemotactic protein 3 (MCP3) in the heart weeks after myocardial infarction re-establishes the ability of infused mesenchymal stem cells to home and engraft into the infarct zone (Penn, 2007). Although subcutaneously administered granulocyte-colony-stimulating factor has been relatively ineffective in mobilizing stem cells of patients after acute myocardial infarction, further studies that define, in detail, the homing process triggered by certain chemokines are clearly needed to improve cell-based gene therapy for the treatment of cardiac dysfunction.

Pluripotent Stem Cells on Trial

Considerable emphasis has been placed on pluripotent stem cells that have the capacity to differentiate into cells derived from any of the three germ layers. Embryonic stem cells are pluripotent stem cells with the greatest potential to differentiate into cardiomyocytes and replace the damaged cardiac muscle. *In vitro* analysis showed that human ESC-derived cardiomyocytes spontaneously contract and generate action potentials of multiple cardiac phenotypes, as well as expression of specific cardiac protein and myofibrillar organization (Passier *et al.*, 2008). *In vivo* ESC-derived cardiomyocytes can function as pacemakers in models of atrioventricular-blocked hearts. In an acute cardiac injury model, human ESC-derived cardiomyocytes formed gap junction and grafted cells behaved as a syncytium. Most studies report functional improvement as apparent at four weeks, whereas one study extended the analysis up to 12 weeks in mice, in which the benefit of the injected cells was no longer present (Passier *et al.*, 2008). Although it cannot be ruled out that paracrine effects are responsible for the increased functional parameters, most likely differences in beating frequency may account for the failure to improve long-term cardiac functions in mouse models (murine heart beats 300–600 times per minute, while human cardiomyocytes beat 60–100 times per minute).

The use of non-autologous human ES cells in a therapeutic context is confounded by their inherent immunogenicity. Numerous studies aimed to reduce immunogenicity after transplantation of host cells have achieved promising results. Mixed chimerism and reversible reactivation of thymic function by analogs of luteinizing-hormone-releasing hormone (LHRH) showed immune tolerance after transplantation of ESCs, providing important proof-of-principle that ES-cell-based therapies are possible in heterologous transplantation experiments (Chidgey *et al.*, 2008). In practice, the advantages of using ESCs are often outweighed by ethical and political considerations, which constitute the chief impediments to the clinical application of these cells. Several strategies to overcome the use of human embryos involve the production of pluripotent stem cells from adult cells. Remarkably, retroviral-mediated overexpression of only four proteins (Oct4, Sox2, Myc, and Klf4) in mouse embryonic or adult fibroblasts generated inducible pluripotent stem-cell-like cells (iPS) able to differentiate into derivatives of the three germ layers (Passier *et al.*, 2008). IPS cells are ideal candidates for autologous transplantation, since production of patient specific iPS cells can theoretically overcome the problem of rejection and immusuppressive therapy.

However promising, both iPS and ESC cells form teratomas if undifferentiated derivatives are present in the pool of injected cells. The carcinogenic nature of ESC and iPS cells limits their therapeutic application and poses an important challenge. Effective differentiation methods need to be developed to yield highly-purified cell populations for transplantation. Although encouraging results in tumor reduction have been obtained using methods to preselect for ESCs that differentiate along a cardiac lineage, or to preserve pluripotency in the absence of Myc for iPS cells, a mere reduction in tumor incidence will not be sufficient to justify clinical trials.

EVOLVING CONCEPTS OF REGENERATION

The limited restorative capacity of the adult mammalian heart has been attributed to the loss of cardiomyocyte versatility soon after birth. Emerging concepts of regeneration

as an evolutionary variable are dramatically illustrated by the relatively robust proliferative capacity of the injured heart in other vertebrate species. The dramatic regeneration of urodele amphibian limb and lens extends to their robust repair of injured myocardium. Unlike their mammalian counterparts, adult newt cardiomyocytes can readily proliferate after injury and contribute to the functional regeneration of the damaged heart. Newts repair their hearts efficiently in response to cardiac damage, leaving none of the dysfunctional scar tissue typical of the post-infarct mammalian myocardium. The recognition that cardiac tissue in other vertebrates can undergo extensive repair has prompted the proposal that regeneration may be a primordial attribute that has been lost during mammalian evolution (Brockes and Kumar, 2002).

Mechanisms of regeneration have been generally viewed as a recapitulation of genetic pathways employed during embryonic development, where tissue stem cells are abundant. Despite similarities in developmental pathways and ultimate morphology of limbs in various tetrapod species, the astonishing capacity to regenerate limbs in amphibians, where the limb develops late in embryogenesis as a semiautonomous module, is not shared by other tetrapods, where limb patterning involves interactions with other parts of the embryo. Relating the relative autonomy and timing of limb development to the regenerative potential of different vertebrate taxa may provide clues to the role of stem cells in adult tissue repair. Indeed, recent studies of newt regeneration point to a mechanism whereby the rebuilding of damaged tissue could couple acute response to injury with local activation of plasticity in surrounding tissues and/or activation of stem cell pools. A transient activity generated by thrombin, a critical component of the clotting cascade that ensures hemostatis and triggers other events of wound healing, has been linked to cell cycle re-entry in multinucleate myotubes during urodele limb regeneration (Brockes and Kumar, 2002). Thus, selective activation of thrombin protease action in response to injury links response to damage and initiation of regenerative growth. It remains to be seen how this mechanism is compromised in mammals, and whether such a linkage can be established in the regenerating myocardium of any species.

Regeneration of the zebrafish heart offers a more genetically accessible model for dissecting the molecular basis of cardiac repair. After surgical removal of the ventricular apex and rapid clotting at the site of amputation, a blastema is formed, comprised of progenitor cells that express precardiac markers, undergo differentiation, and proliferate (Lepilina *et al.*, 2006). Epicardial tissue surrounding the ventricle induces developmental markers and expands rapidly, creating a new epithelial cover for the exposed myocardium. A sub-population of these epicardial cells undergoes epithelial-to-mesenchymal transition (EMT), invades the wound, and provides new vasculature to regenerating muscle (Lepilina *et al.*, 2006). The authors speculate that injury-related signals are insufficient to stimulate meaningful proliferation from existing adult zebrafish cardiac muscle, and that progenitor cells contribute to cardiomyocytes with increased mitotic capacity.

Although the inductive signals triggering epicardial-derived precursor cell contribution to neovascularization in the zebrafish heart have yet to be identified, unexpected clues come from studies of thymosin β4—an endogenously occurring 43 amino acid peptide—in the mammalian heart (Smart *et al.*, 2007). In acutely-infarcted hearts, Tβ4 induces adult epicardial progenitor mobilization and neovascularization, in part through action of its pro-angiogenic cleavage product N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP). *In vitro*, Tβ4 stimulates significant outgrowth from quiescent adult epicardial explants, restoring pluripotency. These studies offer promising clinical prospects for use of Tβ4-induced adult epicardial cells as a viable source of vascular progenitors.

The regenerative potential of the mammalian heart is a rapidly evolving concept. In the near future, cardiac repair is likely to be augmented through a number of avenues (Figure 31-1). The dramatic improvement demonstrated by exogenously administered progenitor cells in both animal and human myocardial repair underscores their therapeutic potential. Although resident cardiac progenitor cell populations have now been identified, the insufficiency of endogenous stem cells to alleviate acute and chronic damage to mammalian cardiac tissue remains to be overcome. Recent advances in our understanding have uncovered an unexpected dynamism in cardiac homeostasis, and highlighted the heterogeneous proliferative potential of resident cardiomyocytes. Enhancing the functional regeneration of

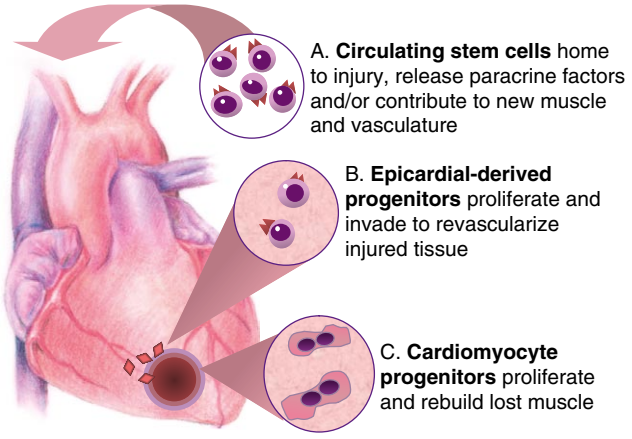


FIGURE 31-1 Modes of cardiac regeneration. Although the pictured endogenous regenerative modes are not mutually exclusive, they provide different possibilities for therapeutic intervention. (A) Circulating stem cells home to the infarcted area, guided by chemoattractive mechanisms, and participate in multiple functions, including neoangiogenesis and myocyte renewal. (B) Injury stimulates activation and proliferation of epicardial progenitors, which participate in neovascularization of injured tissue. (C) Quiescent cardiac stem cells re-enter the cell cycle to replace and rebuild missing tissue.

this most obdurate of organs raises the exciting prospect that regenerative processes in other tissues of the adult mammalian soma might be similarly harnessed to fend off the ravages of aging and disease in a new paradigm of self-renewal.

ACKNOWLEDGMENTS

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KEY WORDS

Cardiac hypertrophy Cellular response of cardiac cells to an increase in biomechanical stimuli, characterized by increased cell size, enhanced protein synthesis and sarcomere organization. Prolonged maladaptive hypertrophy is associated with heart failure.

Fibrosis Formation of fibrous scar tissue after damage of an organ, with complete substitution of the cells constituting the organ with collagenous material. Extended fibrosis leads to organ impairment and loss of functionality.

Regeneration Capacity of certain adult vertebrates to repair damaged organs in a well-defined spatial and temporal plan that reconstitutes the original organ.

Stem cell A cell capable of indefinite self-renewal and differentiation into diverse and contextually specific cell lineages.

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Chapter 32

Potential of Embryonic and Induced Pluripotent Stem Cell Differentiation Culture for Vascular Biology

Jun K. Yamashita, Satomi Nishikawa, and Shin-Ichi Nishikawa

INTRODUCTION

Recent progress in vascular biology has been largely supported by two experimental systems that allow manipulation of *in vivo* angiogenesis; the first is various systems to observe neo-angiogenesis and the second is gene targeting technology. The former has been useful to test both positive and negative molecules that affect the process of angiogenesis, and the latter to characterize the function of a particular gene under normal circumstances. These *in vivo* studies have succeeded in listing a large number of molecules involved in angiogenesis, and in characterizing their functions in various situations, including embryonic vascular development. While this list is still expanding, a new problem that has been recognized is that phenotypes induced by those *in vivo* manipulations are not necessarily sufficient to specify the function of objective molecules, particularly at cell level. Apart from such molecules as VEGF and its receptor, whose role in the development of the vascular system has been specified both at the organism and cell levels, many of the molecules listed so far can be defined as no more than a molecule required for vascular remodeling. An important reason for this problem is the lack of understanding with regard to the function of each molecule at cell level. Indeed, it is difficult to describe how a given molecule is involved in the formation of a complex vascular system without knowing this function. Hence, the development of experimental systems that enable us to evaluate the function of a given molecule at cell level is an essential issue in this field.

Previously, most studies of the cell biology of vascular endothelial cells (ECs) have used EC lines that are generated and propagated *in vitro* by a variety of methods. While those cell lines have been useful for many purposes, it has also been widely recognized that, although many of them are not transformed, they have lost some features that are characteristic of normal ECs. In an attempt to ameliorate the problems of established EC lines, we have sought to prepare relatively normal ECs from ES cells, as ES cells have been proven to differentiate to all somatic cells in our body. We thus expected that ECs, particularly those present in the developing embryo, could be obtained from ES cell differentiation cultures. In this chapter, we will describe our data relating to ECs and their progenitor, which can be prepared in ES cell differentiation culture.

CULTURES FOR EMBRYONIC STEM CELL DIFFERENTIATION

ES cell lines have become actualized by the discovery that LIF can inhibit differentiation of ES cells, while maintaining their proliferation. Hence, induction of ES cell differentiation is achieved by transferring immature ES cells to conditions without LIF. The most popular method for inducing ES cell differentiation has been to use embryoid body (EB) as an environment for differentiation (Doetschman *et al.*, 1985; Burkert *et al.*, 1991; Wiles and Keller, 1991). Using this method, ES cells are first rendered to form aggregates, and then transferred to the LIF− differentiation condition. Two epithelial cell layers, visceral endoderm at the outside and ectoderm at the inside, are spontaneously formed from these aggregates, and various cell lineages have been demonstrated to be generated within EBs, without addition of exogenous cytokines, indicating that EBs can provide a pseudo-embryo-like microenvironment that contains the necessary molecules for ES cell differentiation. Indeed, a two-layered structure with

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visceral endoderm and embryonic ectoderm is reminiscent of the egg-cylinder stage of the embryo.

Although EB culture has advantages, such as easy performance and reproducibility, a clear disadvantage of this method is that it requires a complex three-dimensional (3D) structure that is difficult to manipulate and monitor. To overcome this problem, attempts have been made to induce EB differentiation in a two-dimensional (2D) plane. In an attempt to induce ES cell differentiation in the hematopoietic lineage, Nakano *et al.* (1994) reported a culture of ES cells on OP9 stromal cell line. In this stromal cell-dependent culture, ES cells undergo proliferation and differentiation, without forming 3D structures such as EB. In this culture, ES cells were cultured on OP9 cells which express the molecules required for proliferation and differentiation of hematopoietic stem cells. It is of interest to note that there is a striking variation in the differentiation-supporting ability among stromal cell lines, particularly the repertoire of lineages generated in the culture. Initially, Nakano *et al.* ascribed the activity of OP9 cells to support preferential differentiation to hematopoietic cell (HPC) lineage to the deficit of macrophage colony-stimulating factor (M-CSF) expression in OP9 stromal cell line (OP9 cells are a stromal cell line which was established from *op/op* mouse bearing null-mutation in the M-CSF gene (Yoshida *et al.*, 1990)). However, later study demonstrated that OP9 cells have an activity that induces preferential differentiation of lateral mesoderm, as compared to other stromal cell lines. Indeed, PA6 cells that can also support proliferation and differentiation of hematopoietic stem cells as efficiently as OP9 cells were shown to be defective in inducing the lateral mesoderm (Kataoka *et al.*, 1997). Instead, PA6 cells were shown to be an efficient stromal cell to support neuronal differentiation (Kawasaki *et al.*, 2000). Hence, variation among stromal cell lines will be useful in understanding the molecular mechanisms regulating each distinct cell lineage, and will provide a powerful method to steer ES cell differentiation.

With regard to differentiation of ECs and HPCs, we have shown that the collagen IV matrix is sufficient for ES cell differentiation to these lineages through lateral mesoderm. In this culture, ES cells spread on a 2D plane coated by collagen IV, and undergo differentiation to lateral and paraxial mesoderm (Nishikawa *et al.*, 1998). This result indicates clearly that neither 3D structure nor stromal cells are required for induction of early intermediate stages from ES cells. Our study showed that ES cells spontaneously proliferate and differentiate under a simple culture condition, although the cell density is the critical factor which influences the outcome.

Considering that the next step for ES cell differentiation culture is to develop chemically-defined culture conditions, it is significant that ES cells can undergo differentiation under simple culture conditions. While serum-containing medium can support the differentiation to lateral mesoderm without exogenous growth factors, mesoderm induction does not occur in serum-free medium unless appropriate cytokines, such as BMP, are added. Recently, we established a defined culture condition which can induce a distinct subset of mesoderm cells separately, using ES cell lines capable of distinguishing between the organizer and the middle section of the primitive streak region (Era *et al.*, 2008). We showed that the hematopoietic and endothelial potential of mesoderm were regulated by a balanced combination of BMP4 and activin signaling using this experimental system. It is thus expected that the simplicity of this culture system will be useful in identifying molecular requirements for the induction of each cell lineage.

MARKERS FOR DEFINING INTERMEDIATE STAGES DURING ENDOTHELIAL CELL DIFFERENTIATION

Until now, no ES cell differentiation cultures could steer ES cell differentiation to select a particular pathway at will, although PA6 stromal cell line can induce up to 90% nestin+ cells (Kawasaki *et al.*, 2000). Nonetheless, in all presently available culture methods, including ours, multiple cell lineages are generated simultaneously on induction of differentiation. The most significant problem in this situation is the difficulty of monitoring events in the culture before the appearance of such cells as erythrocytes can be easily characterized. This problem can be ameliorated if intermediate stages in a particular differentiation pathway are distinguished from other cell lineages. While a number of stage- and lineage-specific markers have been developed for defining early intermediate stages, most markers require fixation of cells, and thus are difficult to apply to living cells.

Basically, two methods are available to purify living cells at various intermediate stages. The first method is to use drug-based selection (Li *et al.*, 1998; Marchetti *et al.*, 2002). By this method, drug-resistant genes are expressed under the control of the gene regulatory unit that can direct gene expression at a particular stage, and in a particular cell lineage. After induction of ES cell differentiation, any cells of interest can be selected by adding the appropriate concentration of drug to the culture, if the drug-resistant gene is specifically expressed. The other method is to use cell sorting. In order to apply this technology, cells of interest have to be defined by the expression of cell surface markers that can be detected by labeled antibodies or fluorescent protein, driven by a cell-specific promoter (Nishikawa *et al.*, 1998). In relation to the differentiation of ECs, both methods have been shown to be useful. As any depletion of unwanted cells by drug selection requires some incubation time, drug-based selection is not suitable for the selection of intermediate stages that appear only transiently. Cell sorting technology would be more

useful for this purpose. Moreover, cell sorting technology can combine multiple markers for definition of the cells, which would be difficult to perform by drug-based selection, although it is theoretically possible.

Although multiple markers can be used at the same time as cell sorting, the problem for this application is the absolute deficit in the number of markers available for sorting intermediate stages that appear in early embryogenesis. In this context, we could say that differentiation and diversification of ECs from ES cells might be the best characterized process with multiple markers. As summarized in Figure 32-1, we succeeded in defining distinct differentiation stages of ECs using multiple markers. In this scheme, immature stages of mesoderm differentiation are defined by marker expressions of E-cadherin (ECD, undifferentiated ES cells, embryonic ectoderm, mesendoderm), goosecoid (Gsc, organizer), platelet-derived growth factor receptor-α (αR; mesendoderm, immature mesoderm, paraxial mesoderm), and Flk1 (immature mesoderm, lateral plate mesoderm) (Yasunaga *et al.*, 2005; Izumi *et al.*, 2007; Era *et al.*, 2008). Mesendoderm cells (Gsc+ECD+αR+) give rise to definitive endoderm (Gsc+ECD+Sox17+) and mesoderm (Gsc+ECD−αR+). Immature mesoderm (Flk1+aR+) differentiated into paraxial mesoderm (Flk1−aR+) and lateral plate mesoderm (Flk1+aR−). Immature ECs first appeared as Flk1+VE (vascular endothelral)-cadherin+ CD31−CD34− from Flk1+ mesoderm. Flk1+ mesoderm can also give rise to primitive erythrocytes or vascular mural cells (pericytes and smooth muscle cells) which are negative for Flk1 and VE-cadherin (Yamashita *et al.*, 2000; Fujimoto *et al.*, 2001). Flk1+ mesoderm cells can also give rise to cardiomyocytes (Yamashita *et al.*, 2005). Flk1 promoter/enhancer Flk(p/e)-driven GFP gene and VE-cadherin expression can mark immature ECs that possess hemogenic potential. VE-cadherin+ ECs first appear as a GFP− population, subsequently all ECs become VE-cadherin+/GFP+. Only GFP− ECs can give rise to definitive HPCs, and they are observed in hematopoietic cell clusters in the dorsal aorta, indicating that Flk(p/e)-GFP− ECs represent hemogenic ECs (Hirai *et al.*, 2003). Immature EC populations then successively express CD31, and eventually CD34. Mature ECs are defined as the stage expressing all EC markers, such as Flk1, VE-cadherin, CD31, and CD34. Analysis of the time course of the expression of these molecules demonstrated that they appear successively in an orderly manner. Moreover, when ECs are induced with VEGF and serum, ephrinB2-negative venous ECs are mainly induced. Activation of cAMP pathway together with VEGF induces ephrinB2+ arterial ECs through Notch activation (Yurugi-Kobayashi *et al.*, 2006). When Flk1+ cells are cultured on OP9 for more than three days, prox1+ lymphatic ECs are induced (Kono *et al.*, 2006). This system, thus, can reproduce the sequential cellular processes of ES cell differentiation to various vascular cells and EC diversification (Yamashita, 2004, 2007). Each stage is able to be distinguished and sorted by cell sorter, and the order of each stage during EC differentiation can be confirmed by sorting and short-term culture. As few as two to four days are required for completing all processes after Flk1+ mesoderm appears; dissection of this process can only be obtained by cell sorting. Such a short course is appropriate for transferring this process to a chemically defined condition, thereby determining the molecular requirement for differentiation. Indeed, we have recently demonstrated this process can be supported, to some extent, under serum-free conditions (Hirashima *et al.*, 2003).

UTILITY OF EMBRYONIC STEM CELL CULTURE FOR CELL BIOLOGY OF ENDOTHELIAL CELLS

As a number of reliable methods for inducing ECs from ES cells and markers to purify the cells in this differentiation pathway are available, ECs generated from ES cells are now ready to be used for cell biology.

Clonogenic Assay of Endothelial Cells

An important system which emerged from the investigation of ES cell differentiation culture is a clonogenic assay of the vascular progenitor using OP9 stromal cells. A protocol of this method was described in detail in the report of a method by Hirashima *et al.* (1999). Briefly, the cell population that contains vascular progenitors is seeded onto the OP9 stromal cell layer. After four to seven days of incubation, vascular progenitors form a colony that can be detected by staining with EC specific markers (Figure 32-1). From the single cell deposition analysis, it is likely that this assay can support clonogenic growth of a single progenitor (Nishikawa *et al.*, 1998). Moreover, this assay is effective not only for the progenitors derived from ES cells, but also those present in actual embryos (Fujimoto *et al.*, 2001). Moreover, a similar culture system using OP9 cells has been used for culturing the embryonic fragment to investigate the process of vascular development (Takakura *et al.*, 2000). Induction of ECs from single Flk1+ progenitors is also possible by culturing a single progenitor cell on type IV collagen with the addition of VEGF or other angiogenic factors (Yamashita *et al.*, 2000, Yuvugi-Kobayashi *et al.*, 2006).

It should be emphasized that many stromal cell lines could not be used for this assay. As far as we have tested, no other cell lines could give better results than OP9 cells for this purpose. As no exogenous molecules are required for this assay, it is likely that all essential molecules required for the clonogenic growth of ECs, such as VEGF, are expressed by OP9 cells. Our studies showed that, indeed, many important angiogenic molecules are expressed by OP9 cells (Takakura *et al.*, 1998; Hirashima *et al.*, 1999; Matsumura *et al.*, 2003). Moreover, we think

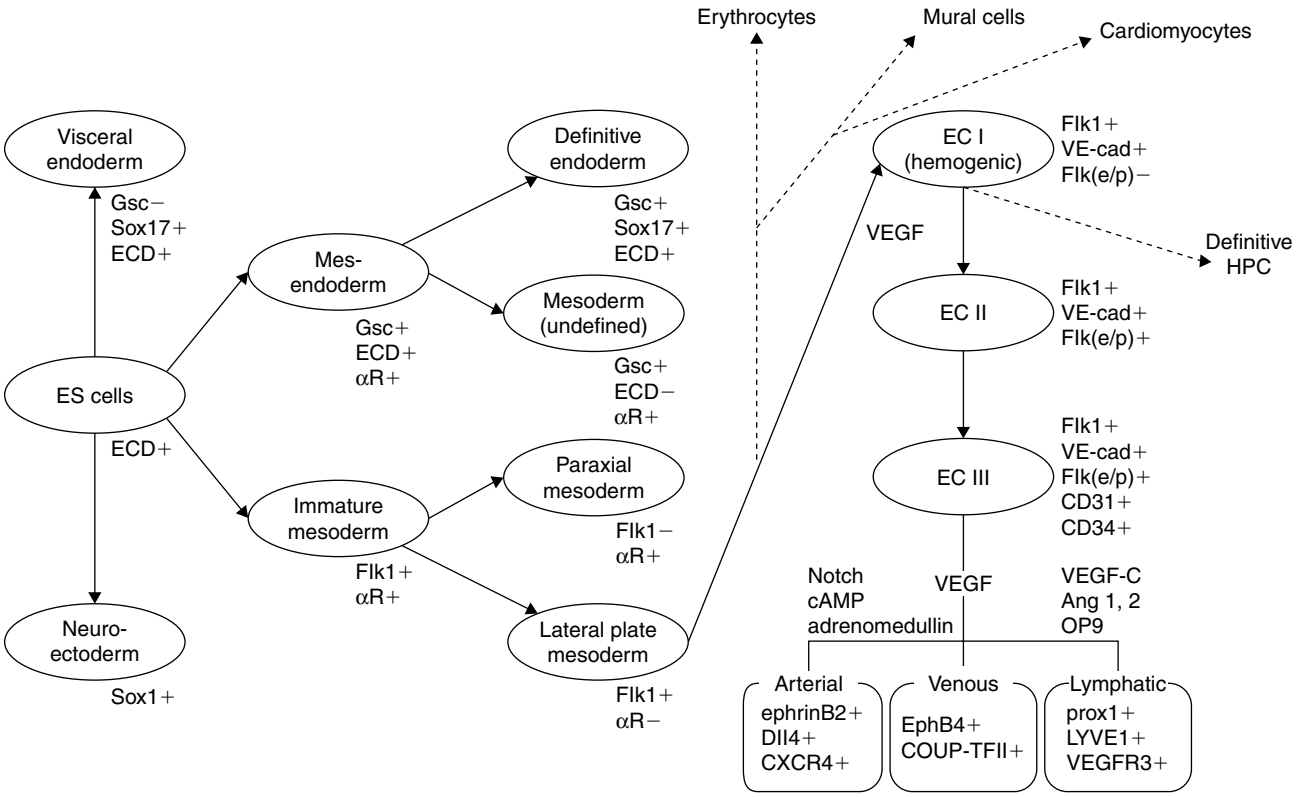


FIGURE 32-1 Intermediate stages during differentiation of endothelial cells from ES cells. Five intermediate stages have been identified during differentiation of endothelial cells from ES cells. Each stage is defined in terms of the expression of surface molecules indicated in this figure.

that the expression level and balance of angiogenic molecules expressed in a given cell line are important factors determining its aptitude for clonogenic assay. Indeed, addition of exogenous VEGF to OP9 culture induces dispersion of the EC colony, rendering the quantification of the EC colony difficult (Hirashima *et al.*, 1999). Nonetheless, OP9 cell lines provide a suitable condition for enumerating the number of clonogenic EC progenitors both from ES cell differentiation cultures and the actual embryos. As the proliferative capacity of ECs may decrease upon maturation, it should be noted that the proportion of EC progenitors clonable by this assay would decrease during embryogenesis.

ENDOTHELIAL CELL DIVERSIFICATION

It has been established that ECs diversify rapidly into different subsets, such as arterial, venous, lymphatic, or capillary ECs, during the development of the vascular system. Recent studies uncovered the molecular network involved in this process, in which Notch and Eph/Ephrin signaling pathways play key roles (Wang *et al.*, 1998; Krebs *et al.*, 2000). As the failure of EC diversification, due to defects of these signal pathways, results in failure in the formation of an organized architecture of vascular network, generation of EC diversity is an essential step for vascular remodeling. While the remodeling defect resulting from null-mutation of those genes is clearly demonstrated in mice bearing null-mutations in those genes, little has been understood concerning the function of each molecule in EC diversification. ES cell differentiation culture, with its inherent potential to deal with differentiating ECs, should be the most suitable experimental system to investigate the process of EC diversification. As shown in Figure 32-1, we have succeeded in inducing arterial, venous, and lymphatic ECs from ES cells, and have demonstrated cellular and molecular mechanisms of EC diversification (Yamashita, 2007). That is, VEGF alone with serum mainly induces venous ECs. Activation of cAMP signaling, together with VEGF, induces Notch activation and arterial ECs. Nevertheless, VEGF and Notch are essential, but not sufficient, to induce arterial ECs. To constructively induce arterial ECs from Flk1+ vascular progenitors *in vitro*, VEGF, Notch, and cAMP signaling are independently required to reconstitute minimum sufficient conditions for arterial EC differentiation (Yurugi-Kobayashi *et al.*, 2006). When Flk1+ cells are cultured on OP9 cells, prox1+/LYVE1+/VEGF receptor-3+ lymphatic ECs appear. In this case, in addition to VEGF-C and angiopoietins, which are essential to induce lymphatic ECs, some humoral factors secreted from OP9 cells should be required to induce lymphatic

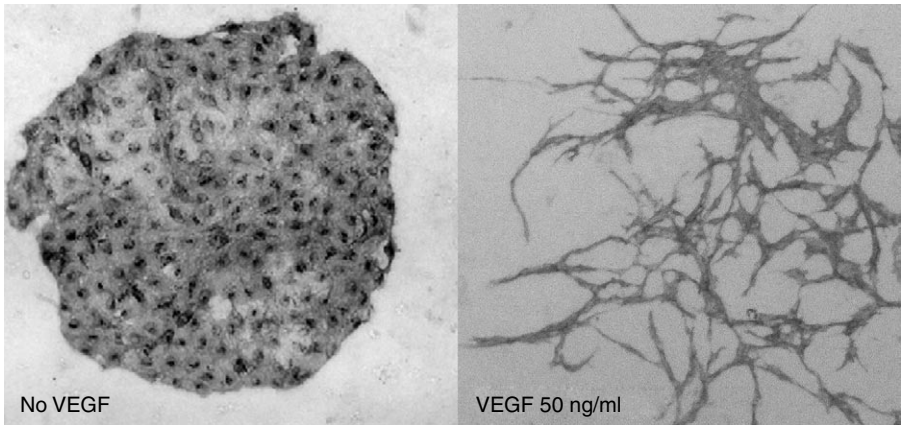


FIGURE 32-2 Clonogenic growth of endothelial progenitors on the OP9 stromal cell layer in the presence or absence of VEGF. Flk1+E-cadherin− lateral mesoderm cells (see Figure 32-1) that were generated *in vitro* from ES cells were sorted and cultured on OP9 stromal cell layer in the presence or absence of VEGF. In the absence of VEGF, the cells grew to form a round colony. Addition of VEGF induces dispersal of endothelial cells. Endothelial cells are identified by CD31 expression.

ECs *in vitro* constructively (Kono *et al.*, 2006). This strategy, thus, can present a combination of minimum sufficient factors for proper cell differentiation by constructively reproducing the differentiation of cells of interest. Our approach should provide new insights into vascular development by adding a novel viewpoint as "constructive developmental biology" (Yamashita, 2007). Genetic animal models have been accepted as one of the most potent and significant experimental strategies in developmental biology. In this, we try to understand the significance of target molecules by comparing the difference in phenotypes between animal models and wild-type controls. Genetic animal models are good at showing the "essential" roles of target genes, but not a minimum "sufficient" condition. By carefully comparing and referring to constructive *in vitro* differentiation models and *in vivo* genetic animal models, these experimental approaches would contribute to further profound understanding of the underlying mechanisms complementarily. It would be easy to understand the importance and potential of "constructive developmental biology" if you imagine induced pluripotent stem (iPS) cells which demonstrate that a combination of four transcription factors (Oct-4, Sox2, Klf4, and c-Myc) are "sufficient" to constructively induce pluripotent cells *in vitro* (Takahashi and Yamanaka 2006; Takahashi *et al.*, 2007).

Cellular Behavior of Endothelial Cells

We expect that the method of preparing ECs in ES cell differentiation culture would have enormous potential in studying the cell biological aspect of EC behavior. While many EC cell lines, such as HUVEC, are available for the cell biology of ECs, it has been recognized that those cell lines have lost properties that are characteristic to ECs during *in vitro* propagation. The extent to which those EC lines represent normal ECs has been a problem to be clarified before using them for cell biology. Another possibility for obtaining normal ECs would be to purify distinct subsets of ECs from developing embryos. Although it is theoretically possible, however, it is not easy to sort a sufficient number of cells from the developing vascular system in embryos. As accumulating evidence suggests that ECs prepared in ES cell cultures maintain many properties of normal ECs, this would be a good method at present available for preparing normal ECs for cell biology. Moreover, as discussed previously, it will be possible to prepare different, distinct, EC subsets with various markers for EC diversity. Some pilot studies of ours and others have already succeeded in indicating the usefulness of this method. As described in the previous section, OP9 cells provide a microenvironment which supports clonogenic proliferation of EC progenitors. A spontaneous balance of angiogenic factors established spontaneously in this culture render EC progenitors to form a round, colony-like, sheet in which cells adhere to each other by VE-cadherin-mediated adherence junction. Thus-formed colony-like EC sheets have become useful in detecting various molecules that enhance the motility of ECs, cell–cell junction formation and cell shape. As shown in Figure 32-2, stable round EC sheets are transformed into clusters of dispersed EC by the addition of exogenous VEGF (Hirashima *et al.*, 1999). Moreover, we showed that VEGFR3 signal inhibits the dispersion of the EC colony, probably negatively modulating the signal pathway of VEGFR2, which enhances EC motility (Matsumura *et al.*, 2003). Addition of an antagonistic mAb to VE-cadherin disrupts EC–EC junction, thereby disturbing the formation of round EC sheets (Hirashima *et al.*, 1999). When this system is combined with various reagents that allow monitoring of different molecules associated with cell–cell junction, more subtle changes in the EC–EC junction can also be detected.

Vascular Structure Formation

Our ES cell differentiation system can reproduce early vascular formation processes in 3D culture (Yamashita *et al.*, 2000). After forming cell aggregates of FACS-purified Flk1+ progenitor cells by floating culture, Flk1+ cell

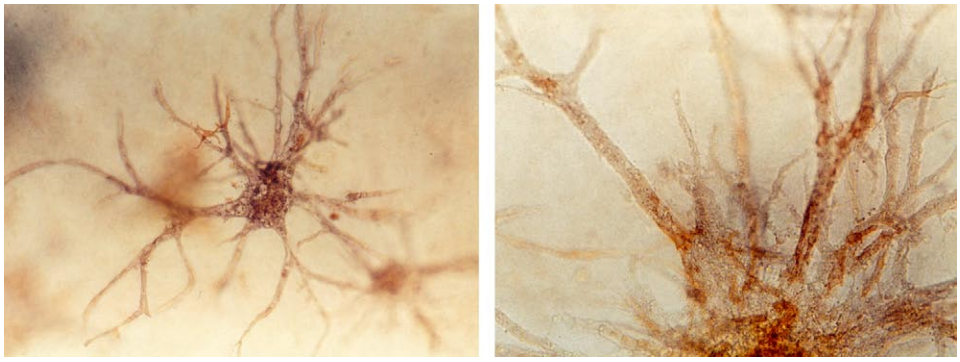


FIGURE 32-3 *In vitro* vascular structure formation from Flk1+ progenitors in 3D culture. Cell aggregates of purified Flk1+ cells are cultured in type I collagen gel. Vascular-like structure of CD31+ ECs (purple) and smooth muscle actin (SMA)+ mural cells (brown) is formed from Flk1+ cell aggregates. Upper panel: gross appearance of a structure. Lower panel: A higher magnification view. Attachment of SMA+ mural cells (brown) to EC tubes is observed.

aggregates are cultured three-dimensionally in type I collagen gel. Cells migrate out from the aggregates, and form vascular-like structure with EC tube supported by mural cells (Figure 32-3). This system should largely mimic early vascular formation processes from the blood islands in yolk sac, including vascular cell differentiation, proliferation, migration, cellular interaction, and structure formation. With the use of this system, it would become possible to assess various aspects of vascular development *in vitro*, even with real-time monitoring. For example, addition of a neutralizing antibody for neuropilin-1 to this 3D culture disrupted vascular formation, but EC differentiation was still preserved (our unpublished observation). Moreover, this system would be useful to screen drugs that may inhibit or enhance vascular formation. Indeed, we have succeeded in identifying novel matrix metalloproteinase inhibitor and HDAC inhibitor, which show anti-angiogenic effects, from marine sponge using this system (Fujita *et al.*, 2003; Nakao *et al.*, 2006).

Use of Human Embryonic Stem and Induced Pluripotent Stem Cells

Human Embryonic Stem Cells

Maintenance conditions, cellular behavior, and differentiation properties differ between mouse and human ES cells. Nevertheless, as for vascular cell differentiation, mouse and human ES cells show largely similar properties. We established a similar sequential EC differentiation system of human ES cells by applying mouse ES cell system. VEGF receptor-2 (VEGFR2; human counterpart of Flk1)-positive/TRA1-60 (undifferentiated human ES cell marker)-negative mesoderm cells appear eight days after the differentiation of human ES cells on OP9 cells. FACS-purified VEGFR2+/TRA1-60− progenitor cells selectively give rise to CD31+ ECs and smooth muscle actin+ mural cells cultured on type IV collagen with VEGF and serum. These progenitor cells also can form vascular-like structure with ECs and mural cells in 3D culture (Sone *et al.*, 2007). Thus, our mouse system for EC differentiation can be almost completely transferred to human studies.

Induced Pluripotent Stem Cells

Recently, a novel pluripotent stem cell line, induced pluripotent stem (iPS) cells, was established from mouse and human somatic cells (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). We applied our ES cell differentiation system to mouse iPS cells, and succeeded in establishing a similar cardiovascular differentiation system using these (Narazaki *et al.*, 2008). Differentiation properties of mouse iPS cells are almost completely identical to those of mouse ES cells. We also showed that the mouse iPS cell differentiation system can be applied to the chemical biology approach for drug discovery using 3D vascular formation system (Nakao *et al.*, 2008). Currently, we are exploring the human iPS differentiation system and anticipating that human iPS cells would show similar differentiation properties as human ES cells. iPS cell technology provides novel approaches and strategies both in basic research and in the clinical field. The reprogramming method would enable dissection of molecular mechanisms of various biological phenomena relating to cell differentiation. Generation of patient-specific iPS cells would contribute to the elucidation of pathophysiology and drug discovery for various diseases (Nishikawa *et al.*, 2008). Thus, extension of our ES cell differentiation technology to other pluripotent stem cells would largely contribute to the progress of vascular biology and its clinical application.

CONCLUDING REMARKS

Here, we have described an *in vitro* system that can induce EC differentiation and vascular formation from ES cells, and also the potential of thus induced EC for cell biological analyses. It is important that this system can offer a novel option to investigate vascular development and regeneration, or other various vascular-related biological

events from a different point of view. Thus, we would like to finish this article by encouraging the readers to consider this system for your research. We are ready to help you to introduce this method for your study.

KEY WORDS

Embryonic stem (ES) cell Cells that are derived from the inner cell mass of the blastocyst and propagated in vitro with their differentiation being arrested. ES cells have been established in mouse, primate, and human, but conditions for maintaining undifferentiated ES cells vary among species.

Fluorescent activated cell sorter A technology extremely useful for purifying intermediates during ES cell differentiation. Gene expression profile is distinct among distinct cell types. As some of such genes characterizing a particular cell type are expressed on the cell surface, fluorescence dye-conjugated antibodies to those molecules can be used for distinguishing and purifying the living cells in terms of expression of a set of molecules on the cell surface.

Vascular endothelial growth factor (VEGF) An essential molecule for proliferation and differentiation of endothelial cells. VEGF binds to both VEGF-receptor (VEGFR)1 and VEGFR2. For inducing endothelial cells in ES cell differentiation cultures, VEGFs have to be added when VEGFR2+ cells appear, which is 3.5 to 4 days after the induction of ES cell differentiation.

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Chapter 33

Cell Lineages and Stem Cells in the Embryonic Kidney

Gregory R. Dressler

INTRODUCTION

The goal of developmental biology is to understand the genetic and biochemical mechanisms that determine cell growth and differentiation, and the three-dimensional patterning of a complex organism. This knowledge can reframe the pathogenesis of human diseases, such as cancer, within a developmental context and can also be applied to create new therapies for the regeneration of damaged tissues. Given the emphasis on stem cells throughout this book, it would seem prudent to discuss the origin of the embryonic kidney with respect to pluripotent renal stem cells, and how they may differentiate into the multiplicity of cell types found in an adult kidney. Unfortunately, no single renal embryonic stem (ES) cells have been conclusively identified. Instead, the kidney develops from a region of the embryo, over a relatively long window of time, while undergoing a sequential anterior to posterior transition that reflects in part the evolutionary history of the organ. The questions that then arise are, at what stage are cells fated to become kidney cells, and when are they restricted in their developmental potential? To address these issues, it is necessary to review the early morphogenesis of the urogenital system. We can then appreciate which genes and molecular markers contribute to early renal development and what cell lineages arise from the region of the embryo devoted to creating the urogenital system.

THE ANATOMY OF KIDNEY DEVELOPMENT

A brief summary of renal patterning and the origin of the renal progenitor cells must begin with gastrulation. In vertebrates, the process of gastrulation converts a single pluripotent sheet of embryonic tissue, the epiblast or embryonic ectoderm, into the three primary germ layers, the endoderm, the mesoderm, and the ectoderm (Figure 33-1A). In mammals, gastrulation is marked by a furrow called the "primitive streak," which extends from the posterior pole of the epiblast. Extension of the primitive streak occurs by proliferation and migration of more lateral epiblast cells to the furrow, followed by invagination through the furrow and migration back laterally under the epiblast sheet. At the most anterior end of the primitive streak is the node or organizer, called Hensen's node in the chick, and the functional equivalent to the blastopore lip or Speeman's organizer in the amphibian embryo. The node is a signaling center that expresses a potent combination of secreted factors for establishing the body axes and left–right asymmetry. The node is positioned at the anterior pole of the primitive streak at approximately the midpoint of the epiblast. The more anterior epiblast generates much of the head and central nervous system, and does not undergo gastrulation in the same manner. Once the streak reaches the node, it begins to regress back to the posterior pole. During this process of primitive streak regression, the notochord is formed along the midline of the embryo and just ventral to the neural plate. The notochord is a second critical signaling center for dorsal ventral patterning of both neural plate and paraxial mesoderm. The axial mesoderm refers to the most medial mesodermal cells which, in response to regression of the streak, become segmented into somites, blocks of cells surrounded by a simple epithelium. At the stage of the first somite formation, going medial to lateral, the notochord marks the midline, the somites abut the notochord on either side, and the unsegmented mesoderm is termed intermediate near the somite and lateral plate more distally (Figure 33-1B). It is this region of intermediate mesoderm within which the kidney will form that is the primary focus of this chapter.

The earliest morphologic indication of unique derivatives arising from the intermediate mesoderm is the formation of the pronephric duct, or primary nephric duct. This single-cell thick epithelial tube runs bilaterally, beginning at around the twelfth somite in birds and mammals. The nephric duct extends caudally until it reaches the cloaca. As it grows, it induces a linear array of epithelial tubules,

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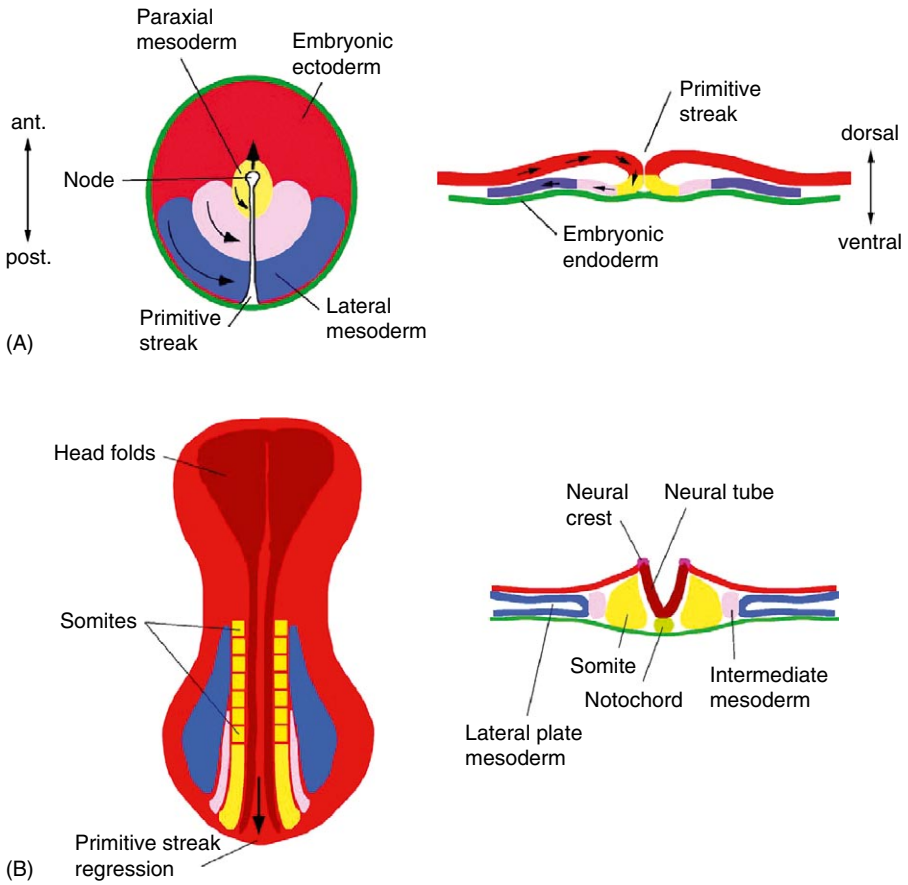


FIGURE 33-1 The origin of the intermediate mesoderm. (A) At gastrulation, cells of the epiblast, or embryonic ectoderm, migrate through the primitive streak. In the mouse, the single sheet of embryonic ectoderm lines a cup-shaped egg cylinder. The left panel is a planar representation of a mouse epiblast looking down into the cup from above. The streak begins at the posterior and moves toward the anterior. Fate mapping studies indicate that lateral plate mesoderm originates from the posterior epiblast, whereas axial mesoderm is derived from more anterior epiblast cells. The intermediate mesoderm most likely originates from cells between these two regions. At the mid-streak stage, the expression of lim1 can already be detected before gastrulation in cells of the posterior epiblast. The schematic on the right is a cross-section through the primitive streak, showing the formation of mesoderm as cells of the epiblast migrate toward the streak, then invaginate, and reverse direction underneath the epiblast sheet. (B) At the time of primitive streak regression, the notochord is formed along the ventral midline. Paraxial mesoderm begins to form segments, or somites, in an anterior to posterior direction. The lateral plate mesoderm consists of two sheets called the somatopleure (dorsal) and the splanchnopleure (ventral). The region between the somite and the lateral plate is the intermediate mesoderm, where the first renal epithelial tube will form.

which extend medioventrally and are thought to derive from periductal mesenchyme (Figure 33-2). The tubules are referred to as pronephric or mesonephric, depending on their position and degree of development, and represent an evolutionarily more primitive excretory system that forms transiently in mammals until it is replaced by the adult or metanephric kidney. Along the nephric duct, there is a graded evolution of renal tubule development, with the most anterior, or pronephric tubules, being very rudimentary, and the mesonephric tubules becoming well-developed with glomeruli and convoluted proximal tubule-like structures. In contrast, the pronephros of the zebra fish larvae is a fully-developed, functional filtration unit with a single midline glomerulus. Amphibian embryos such as *Xenopus laevis* have bilateral pronephric glomeruli and tubules that are functional until replaced by a mesonephric kidney in the tadpole. In fact, it is not altogether obvious in mammals where to draw the distinction between pronephric tubules and mesonephric tubules. Mature mesonephric tubules are characterized by a vascularized glomerulus at the proximal end of the tubule that empties into the nephric duct. The most anterior and posterior mesonephric tubules are more rudimentary, and the most posterior tubules are not connected to the duct at all.

The adult kidney, or metanephros, is formed at the caudal end of the nephric duct when an outgrowth, called the "ureteric bud" or "metanephric diverticulum," extends into the surrounding metanephric mesenchyme. Outgrowth or budding of the epithelia requires signals emanating from the mesenchyme. Genetic and biochemical studies indicate that outgrowth of the ureteric bud is mediated by the transmembrane tyrosine kinase RET, which is expressed in the nephric duct, and the secreted neurotrophin glial derived neurotrophic factor (GDNF), which is expressed

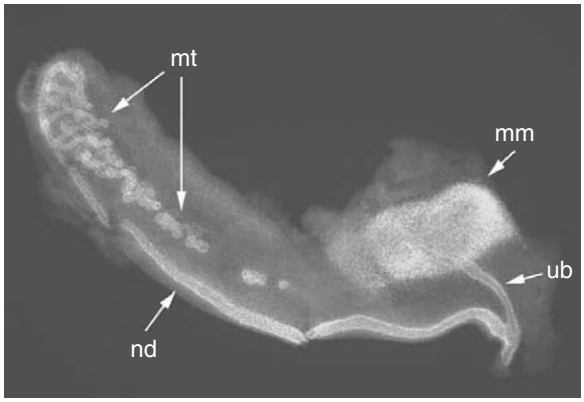


FIGURE 33-2 Expression of Pax2 at the time of metanephric induction. One side of the intermediate mesoderm-derived nephric chord was microdissected from an E11.5 mouse embryo and stained with anti-Pax2 antibodies. The micrograph shows Pax2 in the nephric duct (nd) and the ureteric bud (ub) branching from the posterior duct. Pax2 is in mesonephric tubules (mt), even those more posterior that are not connected to the nephric duct. At the posterior end, Pax2 is in the metanephric mesenchyme cells (mm), surrounding the ureteric bud, which has not yet begun to form epithelia.

in the metanephric mesenchyme. Once the ureteric bud has invaded the metanephric mesenchyme, inductive signals emanating from the bud initiate the conversion of the metanephric mesenchyme to epithelium (Figure 33-3). The induced, condensing, mesenchymal cells aggregate around the tips of the bud. These cells are also referred to as cap mesenchyme, and are likely to represent a distinct lineage. The cap mesenchyme cells will form a primitive polarized epithelium, the renal vesicle. Through a series of cleft formations, the renal vesicle forms first a comma and then an S-shaped body, whose most distal end remains in contact with the ureteric bud epithelium and fuses to form a continuous epithelial tubule. This S-shaped tubule begins to express genes specific for glomerular podocyte cells at its most proximal end, markers for more distal tubules near the fusion with the ureteric bud epithelia, and proximal tubule markers in between. Endothelial cells begin to infiltrate the most proximal cleft of the S-shaped body as the vasculature of the glomerular tuft takes shape. At this stage, the glomerular epithelium consists of a visceral

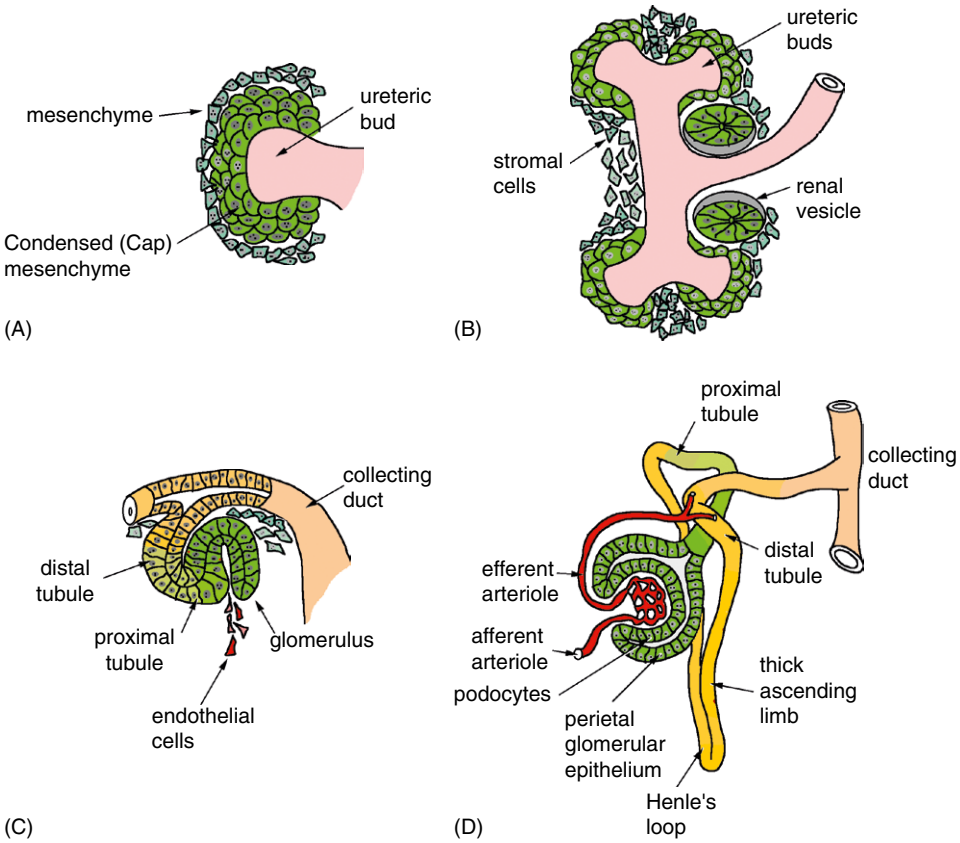


FIGURE 33-3 The sequential conversion of metanephric mesenchyme to renal epithelia. A schematic of the condensation and polarization of the metanephric mesenchyme at the tips of the ureteric bud epithelia is shown. (A) Epithelial precursors aggregate at the tips, whereas stromal cells remain peripheral. This condensed or cap mesenchyme generates all of the epithelial cells of the glomerulus and proximal nephron. (B) The initial aggregates form a primitive sphere, the renal vesicle, as branching ureteric bud epithelia cells extend outward to induce a new aggregate. Stromal cells begin to migrate into the interstitium. (C) At the S-shaped body stage, the mesenchymal-derived structure is fused to the ureteric bud epithelial, which will make the collecting ducts and tubules. The proximal cleft of the S-shaped body is invaded by endothelial cells. Expression of glomerular, proximal tubule, and more distal tubule specific markers can be seen at this stage. (D) The architecture of the nephron is elaborated. Podocyte cells of the visceral glomerular epithelium contact the capillary tuft, and the glomerular basement membrane is laid down. The proximal tubules become more convoluted and grow into the medullary zone to form the descending and ascending limbs of Henle's loop. The distal tubules and collecting ducts begin to express markers for more differentiated, specialized epithelia.

and parietal component, with the visceral cells becoming podocytes, and the parietal cells forming the epithelia surrounding the urinary space. The capillary tuft consists of capillary endothelial cells and a specialized type of smooth muscle cell, termed the "mesangial cell," whose origin remains unclear.

As, or while, these renal vesicles are generating much of the epithelia of the nephron, the ureteric bud epithelia continues to undergo branching morphogenesis, in response to signals derived from the mesenchyme. Branching follows a stereotypical pattern, and results in new mesenchymal aggregates induced at the tips of the branches, as new nephrons are sequentially induced. This repeated branching and induction results in the formation of nephrons along the radial axis of the kidney, with the oldest nephrons being more medullary, with the younger nephrons located toward the periphery. However, not all cells of the metanephric mesenchyme become induced and convert to epithelia; some cells remain mesenchymal and migrate to the interstitium. These interstitial mesenchymal cells, or stromal cells, are essential for providing signals that maintain branching morphogenesis of the ureteric bud and survival of the mesenchyme.

From a stem cell perspective, defining the population of cells that generate the kidney depends, in part, on which stage one considers. At the time of metanephric mesenchyme induction, there are at least two primary cell types, the mesenchyme and the ureteric bud epithelia. Although these cells are phenotypically distinguishable, they do express some common markers and share a common region of origin. As development progresses, it was thought that most of the epithelium of the nephron was derived from the metanephric mesenchyme, whereas the branching ureteric bud epithelium generates the collecting ducts and the most distal tubules. This view has been challenged by cell lineage tracing methods *in vitro*, which indicate some plasticity at the tips of the ureteric bud epithelium so that the two populations may intermingle. Thus, at the time of induction, epithelial cells can convert to mesenchyme, just as the mesenchymal aggregates can convert to epithelia. Regardless of how the mesenchyme is induced, the cells are predetermined to make renal epithelia. Thus, their potential as renal stem cells has begun to be explored. To understand the origin of the metanephric mesenchyme, we begin with the patterning of the intermediate mesoderm.

GENES THAT CONTROL EARLY KIDNEY DEVELOPMENT

Genetic studies in the mouse have provided substantial new insights into the regulatory mechanisms underlying renal development. Although many genes can affect the growth and patterning of the kidney, the genes that control formation of the nephric duct epithelia and proliferation and differentiation of the metanephric mesenchyme are of particular importance with respect to potential renal stem cells (Table 33-1).

Genes that Determine the Nephrogenic Field

From a perspective of stem cells and potential therapy, the adult or metanephric kidney should be a primary focus. However, the early events controlling the specification of the renal cell lineages may be common among the pronephric and mesonephric regions. Indeed, many of the same genes expressed in the pronephric and mesonephric tubules are instrumental in early metanephric development. Furthermore, the earliest events that underlie regional specification have been studied in more amenable organisms, including fish and amphibians, in which pronephric development is less transient and of functional significance.

Although formation of the nephric duct is the earliest morphologic evidence of renal development, the expression of intermediate mesodermal specific markers precedes nephric duct formation temporally, and marks the intermediate mesoderm along much of the anterior–posterior (A–P) body axis. Among the earliest specific markers of intermediate mesoderm are two transcription factors of the Pax family (Figure 33-4), *Pax2* and *Pax8*, which appear to function redundantly in nephric duct formation and extension. The homeobox gene *lim1* is also expressed in the intermediate mesoderm, but is initially expressed in the lateral plate mesoderm before becoming more restricted to intermediate mesoderm. Similarly, the odd-skipped related gene *osr1* marks lateral plate and intermediate mesoderm, before becoming restricted to the intermediate mesoderm.

Genetics implicates all four genes in some aspects of regionalization along the intermediate mesoderm. In the mouse, *osr1* mutants lack metanephric kidneys, but do show nephric ducts more anteriorly. Fate mapping experiments using cre recombinase mediated excision under the control of the *osr1* promoter region demonstrate that many cell types of the kidney are derived from Osr1 positive intermediate or lateral plate mesoderm. These include the epithelial and stromal cells of the metanephric kidney, as well as the vasculature and mesangium, although this type of analysis does not allow one to discern whether a single stem cell gave rise to all of these unique cell types. In *Pax2* mutants, nephric duct formation and extension initiates, but mesonephric tubules and the metanephros are never formed. *Pax2*/*Pax8* double mutants have no evidence of nephric duct formation and do not express the *lim1* gene. Null-mutants in *lim1* also lack the nephric duct, and show reduced ability to differentiate into intermediate mesoderm specific derivatives. The reduced expression of

TABLE 33-1 Genes that Regulate Early Kidney Cell Lineages

Gene	Expression	Mutant Phenotype
Osr1	Lateral plate and intermediate mesoderm	Posterior nephric structures fail to develop
lim1	Lateral plate Nephric duct	No nephric duct, no kidneys
Pax2	Intermediate mesoderm Nephric duct	No mesonephric tubules No metanephros
Pax2/Pax8	Intermediate mesoderm	No nephric duct, no kidneys
WT1	Intermediate mesoderm Mesenchyme	Fewer mesonephric tubules Apoptosis of mesenchyme
Hox11	Metanephric mesenchyme	No metanephros
Eya1	Metanephric mesenchyme	No induction of mesenchyme
Six1	Metanephric mesenchyme	No ureteric bud growth, no induction
Six2	Renal epithelial stem cells	Premature differentiation, loss of self-renewal
wnt9b	Ureteric bud epithelium	Failure to induce the metanephric mesenchyme
wnt4	Mesenchymal aggregates	No polarization of aggregates
bmp7	Ureter bud and metanephric mesenchyme	Developmental arrest postinduction Some branching, few nephrons
FoxD1	Early metanephric mesenchyme Interstitial stroma	Developmental arrest, few nephrons Limited branching
pod-1	Stroma and podocytes	Poorly differentiated podocytes
pdgf(r)	S-shaped body	No vascularization of glomerular tuft

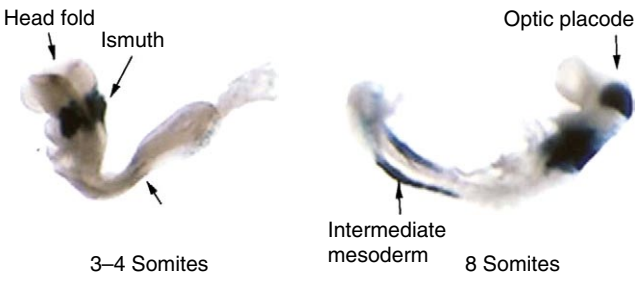


FIGURE 33-4 The activation of Pax2 expression in the intermediate mesoderm. One of the earliest markers for the nephrogenic region Pax2 expression is activated around the four to five somites in the cells between the axial and lateral plate mesoderm. The embryos shown carry a Pax2 promoter driving the *LacZ* gene, and expression is visualized by staining for beta-galactosidase activity. By the eight-somite stage, Pax2 marks the growing intermediate mesoderm even before the nephric duct is formed. Pax2 is also expressed in parts of the nervous system, especially the midbrain–hindbrain junction, known as the rhombencephalic ismuth, and in the optic placode and cup.

Pax2 in *lim1* mutants could explain the inability of these cells to differentiate into urogenital epithelia. In *Pax2*/*Pax8* double-null embryos, however, the lack of Lim1 expression may also be an integral part of the phenotype. Because Lim1 expression precedes Pax2 and Pax8, is spread over a wider area in the pregastrulation and postgastrulation embryo, and is Pax independent, it seems likely that maintenance and restriction of lim1 expression within the intermediate mesoderm requires activation of the *Pax2*/*Pax 8* genes at the 5–8 somite stage. In the chick embryo, ectopic nephric ducts can be generated within the general area of the intermediate mesoderm on retrovirally driven Pax2b expression. These ectopic nephric ducts are not obtained with either lim1 or Pax8 alone, suggesting that Pax2 is sufficient to specify renal tissue. Strikingly, the ectopic nephric ducts paralleled the endogenous ducts, and were not found in more paraxial or lateral plate mesoderm. This would suggest that Pax2's ability to induce duct formation does require some regional competence, perhaps only in the lim1 expressing domain.

If Pax2/Pax8 and Lim1 restriction in the intermediate mesoderm are the earliest events that distinguish the nephrogenic zone from surrounding paraxial and lateral plate mesoderm, the question then remains as to how these genes are activated. In the axial mesoderm, signals derived from the ventral notochord pattern the somites along the dorsal–ventral axis. Similar notochord-derived signals could also pattern mesoderm along the medio–lateral axis. In the chick embryo, the notochord is dispensable for

activation of the *Pax2* gene in the intermediate mesoderm. Instead, signals derived from the somites, or paraxial mesoderm, are required for activation of Pax2, although the nature of such signals is not clear.

In addition to somite-derived signals, additional signals may be required for the formation of epithelia within the predisposed intermediate mesoderm. Activation of Pax2/Pax8 is found more anterior to and precedes formation of the nephric duct. Thus, not all Pax2 positive cells will make the primary nephric duct. In the chick embryo, Bmp4 expressed in the overlying ectoderm is necessary for formation of the nephric duct, which initiates adjacent to somites 10–12, significantly more posterior to the initial Pax2 positive domain.

If Pax2/Pax8 marks the entire nephric region, additional factors must specify the position of elements along the A–P axis in the intermediate mesoderm. Such patterning genes could determine whether a mesonephric or metanephric kidney is formed within the Pax2 positive domain. Among the known A–P patterning genes are members of the *Hox* gene family. Indeed, mice that have deleted all genes of the Hox11 paralogous group have no metanephric kidneys, although it is not clear whether this is truly a shift in A–P patterning, or a lack of induction. However, ectopic expression of Hox11 alleles in a more anterior compartment partially transforms the mesonephric region into a more metanephric phenotype. Thus, posterior Hox genes such as the Hox11 paralogous group are likely to specify the more caudal intermediate mesoderm into assuming a metanephric kidney fate versus a mesonephric fate. A–P patterning of the intermediate mesoderm may also depend on the FoxC family of transcription factors. *Foxc1* and *Foxc2* have similar expression domains in the presomitic and intermediate mesoderm, as early as E8.5. As nephric duct extension progresses, Foxc1 is expressed in a dorso–ventral gradient, with the highest levels near the neural tube and the lower levels in the BMP4 positive ventro–lateral regions. In *Foxc1* homozygous null-mutants, the anterior boundary of the metanephric mesenchyme, as marked by GDNF expression, extends rostrally. This results in a broader ureteric bud forming along the A–P axis, and eventual duplication of ureters. Similar defects are observed in compound heterozygotes of *Foxc1* and *Foxc2*, indicating some redundancy and gene dosage effects. Thus, *Foxc1* and *Foxc2* may set the anterior boundary of the metanephric mesenchyme, at the time of ureteric bud outgrowth, by suppressing genes at the transcriptional level.

Genes that Function at the Time of Metanephric Induction

Pax2 and Pax8 are co-expressed in the nephric duct, but the Pax2 expression domain is broader and encompasses the mesonephric tubules and the metanephric mesenchyme. Thus, Pax8 mutants have no obvious renal phenotype, Pax2 mutants have a nephric duct but no mesonephric tubules or metanephros, and Pax2/Pax8 double mutants lack the nephric duct completely. Thus, either Pax2 or Pax8 is enough for duct formation, but Pax2 is essential for conversion of the metanephric mesenchyme into epithelia.

Pax2 mutants have no ureteric buds, because they do not express GDNF in the mesenchyme, and fail to maintain high levels of RET expression in the nephric duct. Despite the lack of bud, the metanephric mesenchyme is morphologically distinguishable in Pax2 mutants and expresses some specific markers, such as Six2. *In vitro* recombination experiments using Pax2 mutant mesenchyme, surgically isolated from E11 mouse embryos, and heterologous inducing tissues indicate that *Pax2* mutants are unable to respond to inductive signals. Thus, *Pax2* is necessary for specifying the region of intermediate mesoderm destined to undergo mesenchyme-to-epithelium conversion. In humans, the necessity of Pax2 function is further underscored, because the loss of a single *Pax2* allele is associated with renal-coloboma syndrome, which is characterized by hypoplastic kidneys with vesicoureteral reflux.

A second essential gene for conversion of the metanephric mesenchyme to epithelia is *Eya1*, a vertebrate homolog of the *Drosophila eyes absent* gene. In humans, mutations in the *Eya1* gene are associated with branchio-oto-renal syndrome, a complex multifaceted phenotype. In mice homozygous for an *Eya1* mutation, kidney development is arrested at E11, because ureteric bud growth is inhibited and the mesenchyme remains uninduced, although Pax2 and WT1 expression appears normal. However, two other markers of the metanephric mesenchyme, Six2 and GDNF expression, are lost in the *Eya1* mutants. The loss of GDNF expression most probably underlies the failure of ureteric bud growth. However, it is not clear whether the mesenchyme is competent to respond to inductive signals. The *eyes absent* gene family is part of a conserved network that underlies cell specification in several other developing tissues. Eya proteins share a conserved domain, but lack DNA binding activity. The Eya proteins can localize to the nucleus and affect target gene expression through direct interactions with the Six family of DNA-binding proteins. Interestingly, the *Eya1* gene interacts both physically and genetically with *six1*, which is also expressed in the metanephric mesenchyme. *Six1* mutants lack kidneys, due to a failure of ureteric bud invasion and mesenchymal induction. The *six1* gene is also associated with branchio-oto-renal syndrome, which encompasses congenital renal defects, and further underscores the relationship between these two genes.

The Wilms tumor suppressor gene, *WT1*, is another early marker of the metanephric mesenchyme, and is essential for its survival. Expression of WT1 is regulated spatially and temporally in a variety of tissues, and is further complicated

by the presence of at least four isoforms, generated by alternative splicing. In the developing kidney, WT1 can be found in the uninduced metanephric mesenchyme and in differentiating epithelium after induction. Initial expression levels are low in the metanephric mesenchyme, but become up-regulated at the S-shaped body stage in the precursor cells of the glomerular epithelium, the podocytes. High WT1 levels persist in the adult podocytes. In the mouse, *WT1* null-mutants have complete renal agenesis, because the metanephric mesenchyme undergoes apoptosis and the ureteric bud fails to grow out of the nephric duct. The arrest of ureteric bud growth is most probably due to lack of signaling by the *WT1* mutant mesenchyme. As in *Pax2* mutants, the mesenchyme is unable to respond to inductive signals, even if a heterologous inducer is used *in vitro.*

From a stem cell perspective, one of the more interesting genes is *six2*, whose expression is found in the metanephric mesenchyme and the induced, or cap, mesenchyme at the ureteric bud tips. This cap mesenchyme is able to form all of the epithelial cell types of the nephron, and can be considered an epithelial stem cell. In the absence of *six2*, these cap mesenchyme cells precociously undergo differentiation and fail to renew, thus exhausting the supply of epithelial stem cells. Although there is expression of the related *six1* gene, there does not appear to be much redundancy between these genes as the *six1* and *six2* mutant phenotypes are very different. How *six2* maintains the stem cell population is unclear, although the protein may indirectly suppress the inductive mechanisms that promote mesenchymal aggregates to become polarized renal vesicles, thus allowing for proliferation of the uninduced population.

THE ESTABLISHMENT OF ADDITIONAL CELL LINEAGES

At the time of ureteric bud invasion, at least two cell lineages appear to be established: the metanephric mesenchyme and the ureteric bud epithelia. As branching morphogenesis and induction of the mesenchyme progress, additional cell lineages are evident. The early E11.5 mouse metanephros contains precursors for almost all cell types, including endothelial, stromal, epithelial, and mesangial cells. However, it is far from clear whether these cell types share a common precursor, or whether the metanephric mesenchyme is a mixed population of precursors. The latter point may well be true for the endothelial lineage. Although transplantation studies with lineage markers indicate that the vasculature can be derived from E11.5 metanephric kidneys, the Flk1 positive endothelial precursors have been observed to be closely associated with the ureteric bud epithelium, shortly after invasion, and are probably not derived from the metanephric mesenchyme. The lineages most likely to share a common origin within the metanephric mesenchyme are the stromal and epithelial lineages. The maintenance of these two lineages is essential for renal development, because the ratio of stroma to epithelia is a critical factor for the renewal of mesenchyme and the continued induction of new nephrons.

Epithelia Versus Stroma

What early events in the induced mesenchyme separate the stromal lineage from the epithelial lineage? In response to inductive signals, Pax2 positive cells aggregate at the tips of the ureteric bud. Activation of the *Wnt4* gene in these early aggregates appears critical to promote polarization. *Wnt* genes encode a family of secreted peptides that are known to function in the development of many tissues. Mice homozygous for a *wnt-4* mutation exhibit renal agenesis resulting from growth arrest shortly after branching of the ureteric bud. Although some mesenchymal aggregation has occurred, there is no evidence of cell differentiation into a polarized epithelial vesicle. Expression of *Pax2* is maintained, but reduced. Thus, *wnt-4* may be a secondary inductive signal in the mesenchyme that propagates or maintains the primary induction response in the epithelial lineage. The transcription factor FoxD1/BF-2 is expressed in uninduced mesenchyme, and becomes restricted to those cells that do not undergo epithelial conversion after induction. FoxD1 expression is found along the periphery of the kidney and in the interstitial mesenchyme, or stroma. After induction, there is little overlap between FoxD1 and the Pax2 expression domain, prominent in the condensing pretubular aggregates. Clear lineage analysis is still lacking, although the expression patterns are consistent with the interpretation that mesenchyme cells may already have partitioned into a FoxD1 positive stromal precursor and a Pax2 positive epithelial precursor before or shortly after induction. Indeed, analysis of *Osr1* mutations in mouse and zebrafish suggest that *Osr1* is needed to make the choice between stroma and epithelium. Mouse mutants in *FoxD1* exhibit severe developmental defects in the kidney that point to an essential role for *FoxD1* in maintaining growth and structure. Early ureteric bud growth and branching is unaffected, as is the formation of the first mesenchymal aggregates. However, at later stages (E13–14) these mesenchymal aggregates fail to differentiate into comma- and S-shaped bodies at a rate similar to wild-type. Branching of the ureteric bud is greatly reduced at this stage, resulting in the formation of fewer new mesenchymal aggregates. The fate of the initial aggregates is not fixed, because some are able to form epithelium and almost all express the appropriate early markers, such as Pax2, wnt4, and WT1. Nevertheless, it appears that the FoxD1-expressing stromal lineage is necessary to maintain the growth of both ureteric bud epithelium and mesenchymal aggregates. Perhaps factors secreted from the stroma

provide survival or proliferation cues for the epithelial precursors, in the absence of which the non-self-renewing population of mesenchyme is exhausted.

Some survival factors that act on the mesenchyme have already been identified. The secreted transforming growth factor β (TGFβ) family member BMP7 and the fibroblast growth factor-2 (FGF2), in combination, dramatically promote survival of uninduced metanephric mesenchyme *in vitro*. FGF2 is necessary to maintain the ability of the mesenchyme to respond to inductive signals *in vitro*. BMP7 alone inhibits apoptosis, but is not sufficient to enable mesenchyme to undergo tubulogenesis at some later time. After induction, exogenously added FGF2 and BMP7 reduce the proportion of mesenchyme that undergoes tubulogenesis, while increasing the population of FoxD1-positive stromal cells. At least after induction occurs, there is a delicate balance between a self-renewing population of stromal and epithelial progenitor cells, the proportion of which must be well-regulated by both autocrine and paracrine factors. Whether this lineage decision has already been made in the uninduced mesenchyme remains to be determined.

The role of stroma in regulating renal development is further underscored by studies with retinoic acid receptors. Vitamin A deficiency results in severe renal defects. In organ culture, retinoic acid stimulates expression of RET to dramatically increase the number of ureteric bud branch points, thereby increasing the number of nephrons. However, it is the stromal cell population that expresses the retinoic acid receptors (RARs), specifically RARa and RARb2. Genetic studies with *RARa* and *RARb2* homozygous mutant mice indicate no significant renal defects when either gene is deleted. However, double homozygotes mutant for both *RARa* and *RARb2* exhibit severe growth retardation in the kidney. These defects are primarily due to decreased expression of the RET protein in the ureteric bud epithelia and limited branching morphogenesis. Surprisingly, overexpression of RET with a HoxB7/RET transgene can completely rescue the double RAR mutants. Thus, stromal cells may provide paracrine signals for maintaining RET expression in the ureteric bud epithelia. Reduced expression of stromal cell marker FoxD1, particularly in the interstitium of RAR double mutants, supports this hypothesis.

Cells of the Glomerular Tuft

The unique structure of the glomerulus is intricately linked to its ability to retain large macromolecules within the circulating bloodstream, while allowing for rapid diffusion of ions and small molecules into the urinary space. The glomerulus consists of four major cell types: the endothelial cells of the microvasculature, the mesangial cells, the podocyte cells of the visceral epithelium, and the parietal epithelium. The development of the glomerular architecture and the origin of the individual cell types are just beginning to be understood.

The podocyte is a highly-specialized epithelial cell whose function is integral to maintaining the filtration barrier in the glomerulus. The glomerular basement membrane separates the endothelial cells of the capillary tufts from the urinary space. The outside of the glomerular basement membrane, which faces the urinary space, is covered with podocyte cells and their interdigitated foot processes. At the basement membrane, these interdigitations meet to form a highly specialized cell–cell junction, called the "slit diaphragm." The slit diaphragm has a specific pore size to enable small molecules to cross the filtration barrier into the urinary space, while retaining larger proteins in the bloodstream. The podocytes are derived from condensing metanephric mesenchyme, and can be visualized with specific markers at the S-shaped body stage. Although a number of genes are expressed in the podocytes, only a few factors are known to regulate podocyte differentiation. These include the *WT1* gene, which is required early for metanephric mesenchyme survival, but whose levels increase in podocyte precursors at the S-shaped body stage. In the mouse, complete *WT1*-null animals lack kidneys, but reduced gene dosage and expression of WT1 result in specific podocyte defects. Thus, the high levels of WT1 expression in podocytes appear to be required and make these precursor cells more sensitive to gene dosage. The basic helix–loop–helix protein Pod1 is expressed in epithelial precursor cells and in more mature interstitial mesenchyme. At later developmental stages, Pod1 is restricted to the podocytes. In mice homozygous for a *Pod1*-null allele, podocyte development appears arrested. Normal podocytes flatten and wrap their foot processes around the glomerular basement membrane. *Pod1*-mutant podocytes remain more columnar and fail to develop foot processes fully. Because Pod1 is expressed in epithelial precursors and in the interstitium, it is unclear whether these podocyte effects are due to a general developmental arrest because of the stromal environment, or a cell autonomous defect within the *Pod1* mutant podocyte precursor cells.

Within the glomerular tuft, the origin of the endothelium and the mesangium is uncertain. At the S-shaped body stage, the glomerular cleft forms at the most proximal part of the S-shaped body, furthest from the ureteric bud epithelium. Vascularization of the developing kidney is first evident within this developing tuft. Kidneys excised at the time of induction and cultured *in vitro* do not exhibit signs of vascularization, leading to the presumption that endothelial cells migrate to the kidney some time after induction. However, hypoxygenation or treatment with vascular endothelial growth factor (VEGF) promotes survival or differentiation of endothelial precursors in these same cultures, suggesting that endothelial precursors are already present and require growth differentiation stimuli. *In vivo* transplantation experiments using lacZ-expressing

donors or hosts also demonstrate that the E11.5 kidney rudiment has the potential to generate endothelial cells, although recruitment of endothelium is also observed from exogenous tissue, depending on the environment. The data are consistent with the idea that cells within the E11.5 kidney have the ability to differentiate along the endothelial lineage. Presumptive angioblasts are dispersed along the periphery of the E12 kidney mesenchyme, with some cells invading the mesenchyme along the aspect of the growing ureteric bud. At later stages, Flk-1 positive angioblasts localize to the nephrogenic zone, the developing glomerular cleft of the S-shaped bodies, and the more mature capillary loops, whereas VEGF localizes to the parietal and visceral glomerular epithelium. It appears that endothelial cells originate independently of the metanephric mesenchyme, and invade the growing kidney from the periphery and along the ureteric bud.

The mesangial cells are located between the capillary loops of the glomerular tuft, and have been referred to as "specialized pericytes." The pericytes are found within the capillary basement membranes and have contractile abilities, much like a smooth muscle cell. Whether the mesangial cell is derived from the endothelial or epithelial lineage remains unclear. However, genetic and chimeric analyses in the mouse have revealed a clear role for the platelet-derived growth factor receptor (PDGFR) and its ligand platelet-derived growth factor (PDGF). In mice deficient for either PDGF or PDGFR, a complete absence of mesangial cells results in glomerular defects, including the lack of microvasculature in the tuft. PDGF is expressed in the developing endothelial cells of the glomerular tuft, whereas the receptor is found in the presumptive mesangial cell precursors. In ES cell chimeras of *Pdgfr*−/− and +/+ genotypes, only the wild-type cells can contribute to the mesangial lineage. This cell-autonomous effect indicates that signaling from the developing vasculature promotes proliferation and/or migration of the mesangial precursor cells. Also, expression of PDGFR and smooth muscle actin supports a model in which mesangial cells are derived from smooth muscle of the afferent and efferent arterioles during glomerular maturation.

WHAT CONSTITUTES A RENAL STEM CELL?

The issue of renal stem cells is beginning to draw more attention as new information regarding development and lineage specification is unraveled. A prospective renal stem cell should be self-renewing, and able to generate all of the cell types in the kidney. In the simplest terms, all of the cells in the kidney could be generated by a single stem cell population, as outlined in Figure 33-5. If such a single stem cell exists, it is likely to express Osr1 and localize to the early intermediate mesoderm. However, the potential of such cells appears to change over time, as the embryonic kidney transitions from a pronephros to the definitive metanephros. Even at the earliest stage of metanephric kidney development there are multiple identifiable cell types, including the metanephric mesenchyme and the ureteric bud epithelia. Shortly after induction, stromal and epithelial lineages diverge, and the endothelial lineage is evident as Tie2/Flk1+ precursor cells. This interplay between epithelial, stromal, and endothelial precursors is essential for patterning the nephron. If the three earliest cell types can be derived from the metanephric mesenchyme, a single stem cell may indeed exist and continue to proliferate as development progresses. At present, the data suggest that stroma and epithelia may share a common origin, whereas endothelial cells and their potential smooth muscle derivatives constitute a second lineage. Yet, all of these may be derived from the intermediate mesoderm at earlier stages. However, even if there are three separate lineages already demarcated within the metanephric mesenchyme, the most relevant with respect to the repair of renal tissue is the epithelial lineage. Thus, if we consider the possibility of an epithelial stem cell, the following points would be among the criteria for selection: (1) the cells would most likely be a derivative of the intermediate mesoderm; (2) the cells would express a combination of markers specific for the metanephric mesenchyme; and (3) the cells should be able to contribute to all epithelial components of the nephron, *in vitro* and *in vivo.*

Unlike ES cells, it seems improbable that cells from the intermediate mesoderm can be cultured indefinitely without additional transformation or immortalization taking place. Embryonic fibroblasts can be cultured from the mouse, but, in nearly every case, a limited number of cell divisions occur. The problem is apparent even *in vivo*, because the E11 metanephric mesenchyme is essentially quiescent and does not proliferate in the absence of induction. However, growth conditions that mimic induction might be able to allow for the mesenchyme cells to proliferate, while suppressing their differentiation into epithelium. Alternatively, it may be possible to differentiate ES cells into intermediate mesodermal cells using combinations of growth and patterning factors. The culture of nephric bodies using Wnt protein-expressing feeder cells, as developed by Nishinakamura (see Further reading), provides a valuable model for *in vitro* analysis, but has not been able to maintain the pluripotent self-renewal capacity required for a true epithelial stem cell.

At present, the complexity of the kidney still impedes progress in the area of tissue- and cell-based therapies. Not only must the right cells be made, they must be able to organize into a specialized three-dimensional tubular structure capable of fulfilling all of the physiological demands put on the nephrons. Developmental biology can provide a framework for understanding how these cells arise, and what factors promote their differentiation and growth.

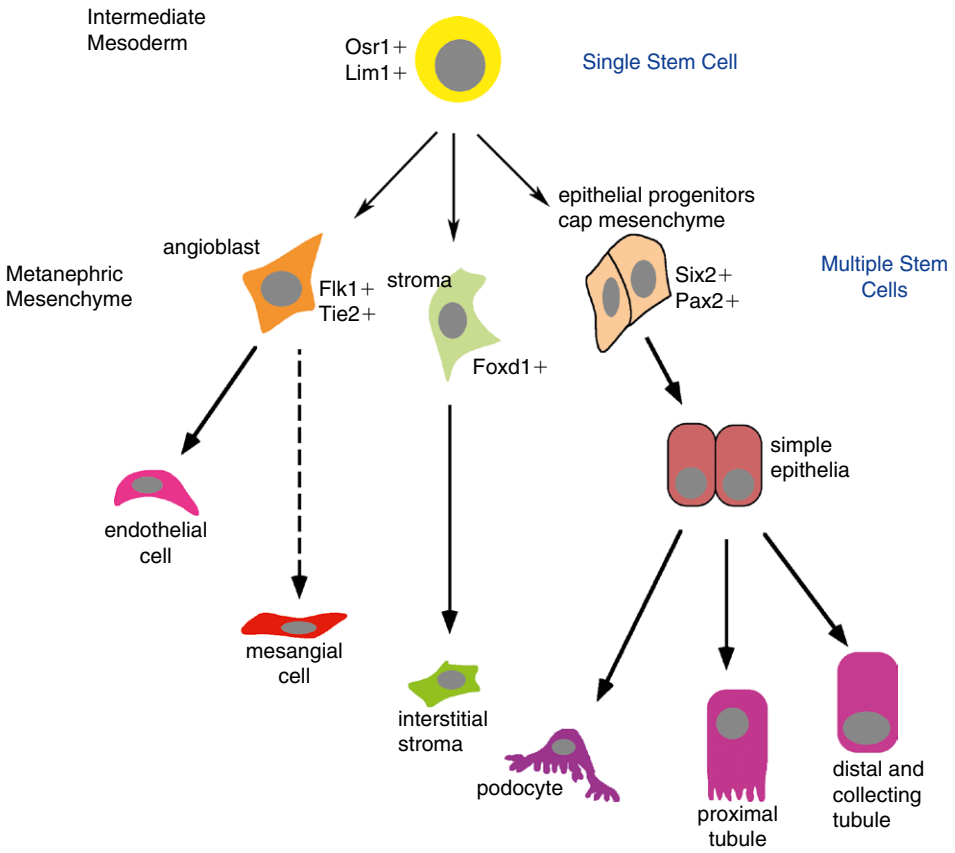


FIGURE 33-5 The major cell lineages of the kidney. Whether the kidney arises from a single renal stem cell or from multiple independent lineages depends in part on what is considered the anlagen or progenitor region. Intermediate mesoderm cells that express Osr1 appear to make almost all of the cell types. However, in the early metanephros, multiple progenitor cells are present. The cell lineage relationships are outlined schematically, with dotted lines reflecting ambiguity in terms of direct lineages. The metanephric mesenchyme contains angioblasts, stromal, and epithelial precursors that express different sets of marker genes, as indicated. Six2 and Pax2 mark the epithelial stem cells, whereas Foxd1 is specific for the stromal cells.

Although we may not be able to make a kidney from scratch, it seems within the realm of possibility that we could provide the injured adult kidney with cells or factors to facilitate its own regeneration. Given the high incidence and severity of acute and chronic renal insufficiency, such therapies would be most welcome indeed.

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KEY WORDS

Intermediate mesoderm A region of mesoderm extending anterior–posterior between the paraxial, or somitic, mesoderm and the lateral plate mesoderm, marked by expression of the *Pax2* and *Pax8* genes.

Kidney induction The activation of the epithelial-specific program in the metanephric mesenchyme in response to signals emanating from the invading ureteric bud.

Metanephric mesenchyme The region of posterior intermediate mesoderm adjacent to the nephric ducts that is able to generate epithelial cells upon induction.

Renal stem cell A hypothetical metanephric mesenchymal cell that could potentially generate all the derivatives found in the nephron, including tubular epithelia, mesangia, stroma, and endothelia.

Renal vesicle The first polarized epithelial derivative of the induced metanephric mesenchyme that is found abutting the branching tips of the ureteric bud.

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Chapter 34

Adult Liver Stem Cells

Markus Grompe

INTRODUCTION

In mammals, the adult liver contains many different cell types of various embryological origins. Nevertheless, the term liver or hepatic stem cell is used only for precursors of the two epithelial liver cell types, the hepatocytes and bile duct epithelial cells, and therefore only hepatocyte and bile duct stem cells will be discussed here.

ORGANIZATION AND FUNCTIONS OF ADULT MAMMALIAN LIVER

To provide background for the discussion of liver stem cell biology, the normal anatomy and function of adult liver will be briefly described. The liver consists of several separate lobes, and represents about 2% of human and 5% of mouse body weight. It is the only organ with two separate afferent blood supplies. The portal vein brings in venous blood rich in nutrients and hormones from the splanchnic bed (intestines and pancreas), and the hepatic artery provides oxygenated blood. Venous drainage is into the vena cava. The bile secreted by hepatocytes is collected in a branched collecting system, the biliary tree, which drains into the duodenum. The hepatic artery, portal vein and common bile duct enter the liver in the same location, the porta hepatis.

The main cell types resident in the liver are hepatocytes, bile duct epithelium, stellate cells (formerly called Ito cells), Kupffer cells, vascular endothelium, fibroblasts, and leukocytes. An adult mouse liver contains about 5×10^7 and an adult human 80×10^9 hepatocytes. The microscopic structure of the liver is essential to the understanding of hepatic stem cell biology. The hepatic lobule (illustrated in Figure 34-1) is the functional unit of the liver. The portal triad, consisting of a small portal vein, hepatic artery branch, and bile duct, is located on the perimeter. Arterial and portal venous blood enter here, mix and flow past the hepatocytes towards the central vein in the middle of the lobule. Liver sinusoids are the vasculature connecting the portal triad vessels and the central vein. Contrary to other capillary beds, sinusoidal vessels have a fenestrated endothelium, thus permitting direct contact between blood and the hepatocyte cell surface. In two-dimensional images, rows of hepatocytes oriented from portal to central form a hepatic plate. A channel formed by adjacent hepatocytes forms a bile canaliculus which drains secreted bile towards the bile duct in the portal triad. Bile secreted by the hepatocytes is collected in bile ducts, lined by duct epithelial cells. The Canal of Hering represents the connection between the bile canaliculi (the inter-hepatocyte space into which bile is secreted) and the bile ducts, at the interface between the lobule and the portal triad. Stellate cells (formerly called Ito cells) represent about 5–10% of the total number of hepatic cells. In addition to storing vitamin A, they are essential for the synthesis of extracellular matrix proteins and produce many hepatic growth factors which play an essential role in the biology of liver regeneration. Kupffer cells also represent about 5% of all liver cells and are tissue macrophages.

The liver is responsible for the intermediary metabolism of amino acids, lipids, and carbohydrates, the detoxification of xenobiotics, and the synthesis of serum proteins. In addition, the liver produces bile, which is important for intestinal absorption of nutrients, as well as the elimination of cholesterol and copper. All of these functions are primarily executed by hepatocytes. The biochemical properties and pattern of gene expression is not uniform among all hepatocytes. The term "metabolic zonation" has been coined to indicate the different properties of periportal (adjacent to the portal triad) and pericentral (adjacent to the central vein) hepatocytes. For example, only pericentral hepatocytes express glutamine synthase and utilize ammonia to generate glutamine. In contrast, periportal hepatocytes express urea cycle enzymes and convert ammonia to urea.

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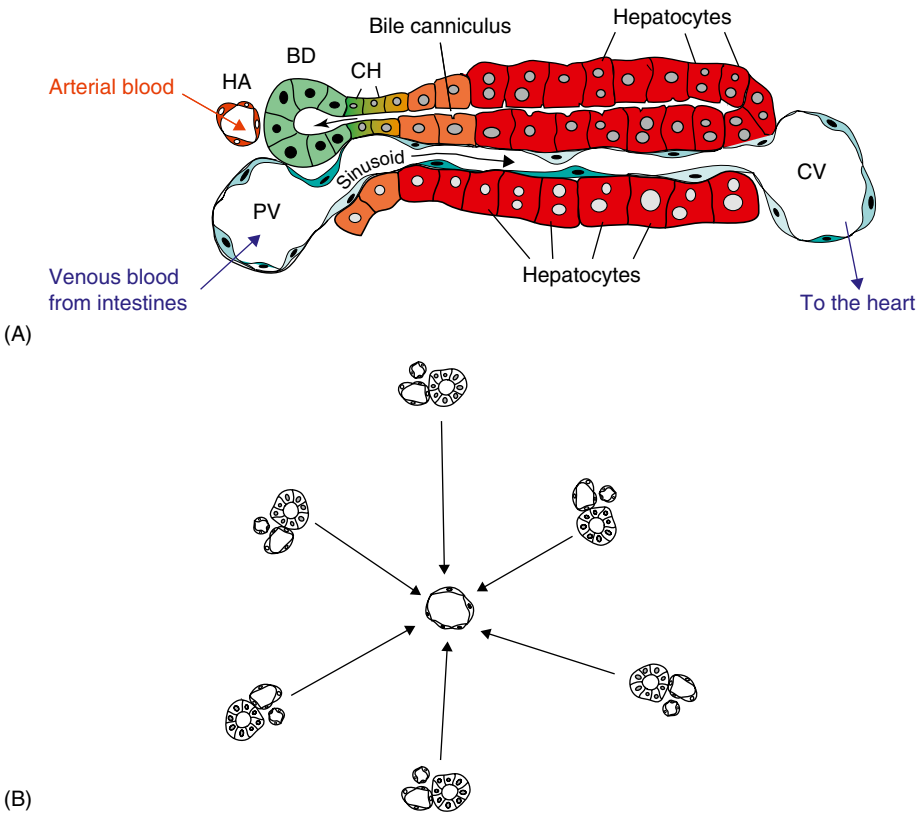


FIGURE 34-1 Structure of the hepatic lobule. (A) The portal triad consists of bile ducts (BD), hepatic artery (HA) and portal vein (PV). Mixed blood from the hepatic artery and portal vein flows past hepatocytes through the sinusoids, covered with fenestrated endothelial cells to the central vein (CV). Bile produced by the hepatocytes is collected in the bile canniculus and flows towards the bile duct. The Canal of Hering (CH) is the junction between the hepatic plate and the bile ducts. This is the region where oval cell precursors reside. (B) Each hepatic lobule consists of one central vein and six surrounding portal triads.

LIVER STEM CELLS

The liver is known to have a very high capacity for regeneration. Mammals (including humans) can survive surgical removal of up to 75% of the total liver mass. The original number of cells is restored within one week and the original tissue mass within two to three weeks. Importantly, liver size is also controlled by prevention of organ overgrowth. Hepatic overgrowth can be induced by a variety of compounds, such as hepatocyte growth factor (HGF) or peroxisome proliferators, but the liver size returns to normal very rapidly after removal of the growth inducing signal. The role of liver stem cells in regeneration has been controversial, but many of the apparent inconsistencies can be reconciled by considering the different definitions which have been used for these cells. Current evidence strongly suggests that different cell types and mechanisms are responsible for organ reconstitution, depending on the type of liver injury. In addition, tissue replacement by endogenous cells (regeneration) must be distinguished from reconstitution by transplanted donor cells (repopulation). Thus, the definition of liver stem cells includes: (1) cells responsible for normal tissue turnover; (2) cells which give rise to regeneration after partial hepatectomy; (3) cells responsible for progenitor-dependent regeneration; (4) cells which produce in hepatocyte and bile duct epithelial phenotypes *in vitro*; and (5) transplantable liver repopulating cells. In the following text, liver stem cells will be discussed according to each of these definitions.

Cells Responsible for Normal Liver Tissue Turnover

The average life span of adult mammalian hepatocytes can be estimated to be ~200–300 days. The mechanism by which these cells are replaced has been of interest for some time. One important earlier model regarding normal liver turnover was termed the "streaming liver." According to this model, normal liver turnover is similar to regeneration in the intestine, with young hepatocytes being born in the portal zone and then migrating towards the central vein. The different patterns of gene expression in periportal and pericentral hepatocytes were explained by the maturation process during this migration, and thus represented a typical lineage progression. However, strong evidence against the streaming liver hypothesis now exists. First, it has been shown that the gene expression pattern in hepatocytes is crucially dependent on the direction of blood flow. If blood flow was reversed such that portal blood entered the lobule through the central vein and exited via the portal vein, the pattern inverted. Therefore, the lobular zonation is best explained by metabolite-induced gene regulation, not lineage progression. Second, retroviral marking studies

provide clear evidence against any hepatocyte migration during normal turnover. These results have also been confirmed by utilizing the mosaic pattern of X-inactivation in female mice to analyze patterns of hepatocyte growth. Together, current evidence clearly indicates that normal liver turnover in adult animals is mediated primarily by *in situ* cell division of hepatocytes and bile duct epithelial cells themselves, and not stem cells.

Cells Which Give Rise to Regeneration after Partial Hepatectomy

The process of liver regeneration after partial hepatectomy has been well-studied, and is the subject of several excellent reviews. During partial hepatectomy, specific lobes are removed intact without damage to the lobes left behind. The residual lobes grow to compensate for the mass of the resected lobes, although the removed lobes never grow back. The process is completed within one week. Again, as in normal liver turnover, there is no evidence for the involvement of or requirement for stem cells in this process. Classic thymidine-labeling studies show that virtually all hepatocytes in the remaining liver divide once or twice to restore the original cell number within three to four days. The earliest labeled hepatocytes are seen 24 hours after partial hepatectomy, with the peak of thymidine incorporation occurring after 24–48 hours, depending on the species. Interestingly, there is zonal variation, depending on how much tissue is removed. When only 15% of the liver is surgically removed, periportal (zone 1) hepatocytes divide preferentially, whereas cell division is seen equally in all three zones after 75% partial hepatectomy. Following the hepatocytes, the other hepatic cell types also undergo a wave of mitosis, thereby restoring the original number of all liver cells within seven days.

Many positive and negative factors important for the initiation of regeneration after partial hepatectomy have been identified. The most important ones are hepatocyte growth factor (HGF), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), transforming growth factor-alpha (TGF-α), and epidermal growth factor (EGF). Non-peptide hormones also have a significant role in the regenerative response after liver injury. Triiodothyronine and norepinephrine can stimulate hepatocyte replication *in vivo*. It is currently unknown whether any of these factors are also important for progenitor-dependent liver regeneration or engraftment and expansion of liver stem cells (see below).

Less knowledge exists about the mechanisms by which hepatocyte cell division and liver regeneration are stopped after the appropriate liver mass has been restored. In particular, the exogenous signals (endocrine, paracrine, or autocrine) which are involved in sensing the overall liver cell mass and negatively regulating its size are not known. Some evidence suggests that transforming growth factor-beta 1 (TGF-β1) may be important in the termination of liver regeneration. Recent work has strongly implicated bile acid levels in blood as regulators of liver growth. In addition, mammalian genes comparable to those in the *Drosophila* Hippo kinase signaling cascade, which regulate wing mass during development, can also control hepatocyte proliferation. When YAP, the mammalian counterpart to Yorki, the last gene in the *Drosophila* Hippo kinase cascade, is overexpressed in a transgenic mouse model, hepatocyte proliferation becomes unchecked, and there is massive liver hyperplasia and hepatic carcinogenesis. When YAP hyperexpression is turned-off or blocked, liver size returns to normal. It is therefore possible that the hippo kinase pathway plays a decisive role in determining overall liver size.

Cells Responsible for Progenitor-dependent Regeneration

Oval Cells

Although neither cell replacement during normal tissue turnover, nor after injury by partial hepatectomy, requires stem cells for organ regeneration, this is not true for all types of liver injury. In some types of damage to liver, small cells with a high nuclear/cytoplasmic ratio emerge in the portal zone, proliferate extensively, and migrate into the lobule. These small cells, which eventually become differentiated hepatocytes, are termed "oval cells" because of their typical morphology in the rat. Although the term "oval cells" is widely used in the field, it is important to emphasize that this term is used for a highly-heterogeneous population of cells. No uniformly accepted definition exists, and thus it is not surprising that reports on the properties of "oval cells" are sometimes conflicting. Not only do multiple different cell types emerge in livers undergoing "oval cell" activation, but it is also not clear whether "oval cells" from different animal species or from different hepatic injuries are comparable. The marker reagents to better understand heterogeneity within these injury-activated populations are only now becoming available. In the future, it is likely that the term "oval cell" will be replaced by more specific designations, based on expression of such clearly defined markers.

Currently, however, there is no complete consensus regarding the origin and properties of these "oval cells". Nonetheless, one important point of agreement is that "oval cells" are not derived from hepatocytes, but instead are the offspring of a cell in the Canal of Hering (Figure 34-1). "Oval cell" proliferation therefore represents an example of progenitor-dependent liver regeneration, and the cell which gives rise to oval cells can be considered a "facultative liver stem cell." It is important to emphasize that the "oval cells" themselves are not the stem cells, but represent an intermediate stage between the stem cell and the differentiated lineages, i.e., hepatocytes and bile ducts. In the rat, chronic liver

TABLE 34-1 Induction of Progenitor-dependent Liver Regeneration

Species	Chemical/Manipulation
Mouse	Dipin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) Phenobarbital + cocaine + p.H. Choline-deficient diet + DL-ethionine High-fat diet + ethanol
Rat	2-Acetylaminofluorene (AAF) Diethylnitrosamine (DEN) Solt-Farber model DEN + AAF + p.H. Modified Solt-Farber model AAF + p.H. Choline-deficient diet + DL-ethionine D-galactosamine + p.H. Lasiocarpine + p.H. Retrorsine + p.H.

p.H. = partial hepatectomy

injury by chemicals such as DL-ethionine, galactosamine, and azo dyes represent examples of this type of liver damage (see Table 34-1). The toxic drugs are often combined with surgical partial hepatectomy. Many of the compounds which induce oval cell proliferation are DNA damaging agents or carcinogens, and oval cells can therefore be considered precancerous. Clinically, the most important condition associated with "oval cell activation" is steatohepatitis, in which the hepatocytes become filled with fat vesicles. Alcoholic liver disease falls into this category, and progenitor activation has been reported in both mice and humans with this disorder. A common feature of progenitor-dependent liver regeneration is that the hepatocytes themselves cannot divide normally. Thus, progenitor-dependent regeneration may be utilized when parenchymal hepatocytes are severely damaged on a chronic basis, and/or are unable to regenerate efficiently.

Table 34-1 shows a list of conditions that result in oval cell proliferation in the rat and mouse. In the rat, oval cells express markers of both bile-duct epithelium (CK19), and hepatocytes (albumin). They also express high levels of α-fetoprotein, and are thus similar to fetal hepatoblasts in their gene expression profile. Classically, oval cells have therefore been considered to be bi-potential (capable of producing both hepatocytes and bile ducts) precursors to mature epithelial liver cells. However, most of the support for oval cell bi-potentially comes from *in vitro* differentiation of immortal cell lines derived from livers undergoing the oval cells response. In contrast, good evidence exists for the notion that oval cells can be hepatocyte precursors *in vivo*. For example, pulse-labeling with ^{3}H-thymidine, and following the progression of the label, was used to demonstrate that oval cells in rats differentiated into distinct clusters of basophilic hepatocytes. A very recent study in mice is the first to use genetic lineage tracing of oval cells. A floxed lacZ reporter under the control of the Foxl1 transcription factor was used. Oval cell activation led to the *de novo* emergence of Foxl1 positive cells in the periportal region, and lineage tracing showed that Foxl1 positive precursors could produce both hepatocytes and bile ducts. However, the study did not address whether the same cell clonally produced both cell types. Formally, it remains possible that there are separate progenitors for bile epithelium and hepatocytes. Because of their similarity to hepatoblasts in the rat, and their probable bi-potential capacity, oval cells have been considered early progenitors in analogy to committed hematopoietic progenitors. Thus, oval cell precursors located in the Canal of Hering represent likely candidates to be liver repopulating stem cells.

In the rat, several monoclonal antibodies have been raised against oval cells and used to study lineage progression. The marker OV6 has found wide application in a variety of studies. Generally speaking, studies with these reagents have confirmed the similarity between oval cells and fetal hepatoblasts in rats.

Recently, several protocols have been developed which result in progenitor-dependent hepatocyte regeneration in the mouse. One particularly useful regimen utilizes the chemical 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Mouse oval cells differ from their rat and human counterparts by not expressing α-fetoprotein (AFP). OV6 also does not react with murine oval cells, and until very recently only one oval cell-specific antibody, termed A6, had been developed for murine oval cells. A panel of new surface markers raised against cells present in the murine oval cell response has demonstrated extensive antigenic heterogeneity among hepatic cells activated by DDC. At least two distinct classes of proliferating epithelial cells can be observed, those within ducts and cells that are immediately periductular. Similarly, rat oval cells can be fractionated according to their cell surface expression of OV1, revealing sub-populations with clearly distinct biological behavior. All of these findings emphasize the heterogeneity of oval cell populations, and highlight that future studies are needed to determine the biological relevance of the distinct cell classes.

Oval cell proliferation has also been described in a variety of human liver diseases, indicating that progenitor-dependent regeneration can be found in multiple organisms. Oval cells are found in disorders associated with chronic liver injury, and are located at the edges of nodules in liver cirrhosis. In both the rat and in humans OV6 is a useful marker for these cells.

Interestingly, rat oval cells induced by a classic carcinogen regimen express multiple genes typically associated with hematopoietic stem cells. The first of these to be identified was stem cell factor (SCF), and its cognate receptor, the c-kit tyrosine kinase. Isolation of hematopoietic stem cells by fluorescence-activated cell sorting (FACS) uses c-kit antigen as an important positive marker. Both the

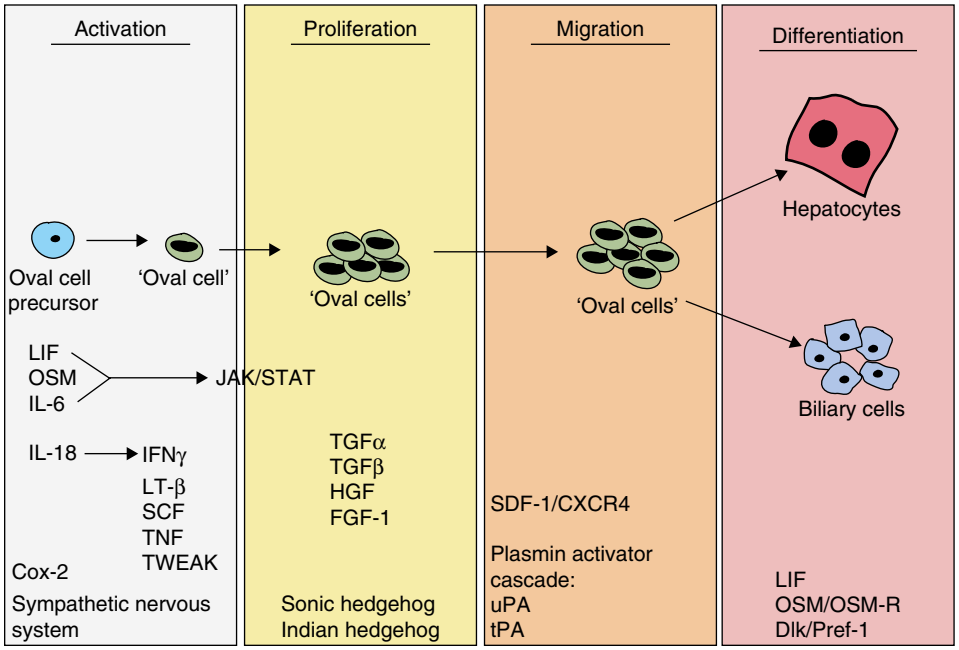


FIGURE 34-2 Signaling events during the hepatic oval cell response. A timeline representing the stages of oval cell activation: activation, proliferation, migration, and differentiation. The factors that are involved in each stage of the response are listed at the bottom.

SCF and the c-kit genes were expressed during the early stages of oval cell proliferation after partial hepatectomy in the 2-acetylaminofluorene (AAF)/partial hepatectomy model, while neither simple partial hepatectomy nor AAF administration alone induced a noticeable expression of the SCF/c-kit system. *In situ* mRNA hybridization revealed that the c-kit transcripts were restricted to AFP-expressing oval cells, whereas the SCF transcripts were expressed in both oval and stellate cells. In addition, some studies have claimed that the Thy-1 marker also used for sorting of HSC in mouse was also highly-expressed in rat oval cells. More recent studies, however, indicate that Thy-1 is not a marker for liver repopulating oval cells.

The expression of HSC markers in oval cells is not unique to the rat. Human oval cells isolated from patients with chronic biliary diseases were found to express CD34, as well as the bile duct marker cytokeratin 19 (CK-19). In addition, cells which are c-kit positive, but negative for hematopoietic markers, have been identified in human pediatric liver disease. Very recently, very high levels of Sca-1 (stem cell antigen) were reported to be expressed on murine oval cells induced by DDC. Therefore, multiple independent studies support the concept that hepatic oval cells, but not regenerating hepatocytes, can express genes also found in HSC. These findings have resulted in the hypothesis that oval cell precursors may ultimately be bone marrow derived (see below).

Molecular Regulation of the Oval Cell Response

The oval cell response can be divided into four phases: activation; proliferation; migration; and differentiation.

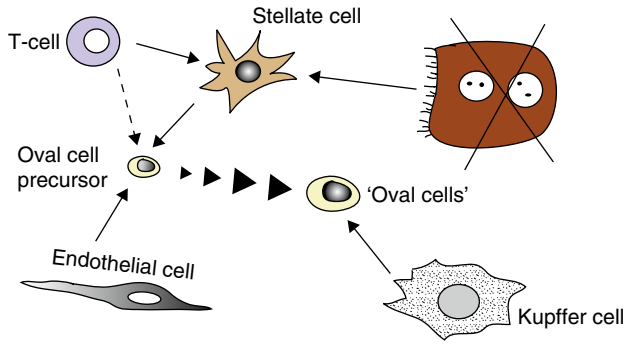


FIGURE 34-3 Cells involved in the hepatic progenitor response. Multiple epithelial and non-epithelial cell types proliferate and/or become activated during the oval cell response, which is initiated by stress/damage to mature hepatocytes. Hypothetical signaling events are indicated by arrows. Cytokines released by inflammatory cells (T-cells) at the site of injury may stimulate "oval cell" proliferation either directly through the oval cell precursor or indirectly via stellate cells. Macrophages and endothelial cells may also be involved.

Most of the details regarding the molecular regulation of hepatic progenitor activation currently remain unknown. However, genetic studies in the mouse and *in vitro* studies of oval cell lines in culture have revealed some factors that impact different aspects of the process. Figure 34-2 summarizes the molecules that are currently known. The most well-established factors will be discussed briefly here, and have been extensively reviewed by us (see Further Reading). It is important once again to highlight the cellular heterogeneity of hepatic progenitor activation (Figure 34-3). Several different epithelial, hematopoietic, and mesenchymal cell types are present, and it is in many cases unclear which cells are producing the different factors, and which are receiving the signals. Factors that impact the

oval cell response may do so either directly (acting on the proliferating epithelial cells), or indirectly by signaling to a non-epithelial cell type which may in turn then act upon the oval cells or their progenitors. In future studies, it will be important to dissect the detailed cellular topology of the process, as well as the kinetics, i.e., the temporal sequence of signaling events.

Several lines of evidence demonstrate the importance of IFN-γ in oval cell activation. IFN-γ-deficient mice have an attenuated oval cell response, while liver regeneration after partial hepatectomy is enhanced. Further, IFN-γ in conjunction with tumor necrosis factor (TNF) and lipopolysaccharide, which are both present during liver growth, causes cell cycle arrest in cultured hepatocytes, while activating oval cell growth. This differential effect on hepatocytes and oval cells has also been reported for TGF-β. TGF-β induces apoptosis in mature hepatocytes, but does not affect oval cells or bile-duct epithelium. Thus, one of the themes beginning to emerge regarding hepatic progenitor activation is inflammatory cytokines that have opposite effects on hepatocytes and oval cell precursors.

IL-6 appears to impact both activation and proliferation of oval cells, in that IL-6-deficient mice have a blunted response to a choline deficient diet. Similarly, TNF-α levels are very high in the early phases of oval cell activation. Mice with a deficiency of the TNF receptor 1 develop very few oval cells, whereas TNFR2 knockout mice have a normal oval cell response.

One pathway has been associated with the emergence of oval cells even in the absence of injury. Transgenic mice expressing Tweak in hepatocytes display an "oval cell response." The proliferating ducts express Fn14, the receptor for this TNF-like ligand. Whether Tweak is important for progenitor activation during injury has not yet been determined.

Elegant recent work suggests an important role for hedgehog signaling in progenitor activation, particularly in alcoholic liver disease. In both mice and humans alcoholic steatohepatitis results in the proliferation of immature ductular cells in the portal tracts. This is accompanied by transcriptional up-regulation of hedgehog mRNAs (Shh and Ihh), and expression of hedgehog target genes in the proliferating ducts, as well as the adjacent myofibroblasts. Hedgehog inhibitors can impair progenitor proliferation. Indirect evidence suggests that activation of hedgehog signaling may be downstream of TGF-β, i.e., the inflammatory response.

Cells Which Produce Hepatocyte and Bile Duct Epithelial Phenotypes *In Vitro*

Several *in vitro* models for hepatic stem cell growth and differentiation have been developed. Two general approaches to the *in vitro* study of liver stem cells can be distinguished. First, cell sorting can be used to isolate putative liver stem cells directly from the liver by cell surface markers. These cells can then be cultured, and their growth and differentiation potential can be assayed *in vitro*. Second, immortal cell lines can be used to study hepatic growth and differentiation. Hepatic progenitor cell lines can be derived from liver tissue by extensive *in vitro* manipulation and growth. In addition, there has recently been much effort invested in the targeted differentiation of pluripotent stem cells towards the hepatocytic lineage. To date, only a limited amount of work has been done on the prospective isolation of hepatic progenitors by cell sorting. In contrast, putative liver progenitor cell lines from several mammalian species have been isolated and propagated in tissue culture, including mouse, rat, pig, and human. These *in vitro* systems have both scientific and potential medical uses. The medical purpose is to generate large numbers of hepatocytes *in vitro* for therapeutic transplantation. The scientific aims are to understand the factors which control the differentiation of these cells into hepatocytes and biliary duct epithelium.

In addition to the creation of hepatocyte progenitor cell lines, several investigators have devised strategies to conditionally immortalize differentiated hepatocytes for *in vitro* expansion and subsequent transplantation. To date, the only cells lines which have had documented therapeutic effects in animal models have been these cell lines, or primary cultures derived from hepatocytes themselves.

Prospective Isolation of Hepatocyte Progenitors by Cell Sorting

The isolation of putative progenitors from the liver requires an assay that can be used to determine whether a cell has progenitor activity. Several laboratories have now reported colony-forming assays for hepatic epithelial cells for several species, including mouse and human. The colonies can be grown with serum-free media, facilitating studies of the effects of individual factors. ECFC (epithelial colony forming cells) were first isolated from fetal mouse liver. The cells were fractionated by FACS and analyzed for their proliferative, as well as differentiation, capacity. A multipotent population of cells was identified in ED 13.5 mouse liver. These cells were characterized as c-kit−, CD45−, c-met+, Ter119−, CD49f+/low. *In vitro* they proliferated extensively, and could be differentiated into either hepatocytic or biliary lineages. However, the same cells could also express multiple pancreatic markers when cultured long-term or transplanted into a pancreatic environment *in vivo*. The genes expressed *in vitro* included endocrine markers (preproinsulin, preproglucagon), pancreas specific transcription factors (pdx-1, pax6), and exocrine markers (amylase-2, lipase). The same investigators used a similar

sorting approach for neonatal mouse pancreas. The c-kit−, CD45−, c-met+, Ter119−, CD49f+/low cells from pancreas were able to be differentiated into hepatocytes *in vitro*. Interestingly, adult liver cells did not yield any colonies under the same conditions, which readily produced them with fetal and neonatal cells. New growth conditions had to be developed and were reported recently. Two types of colonies can form with cells DDC-treated liver undergoing oval cell activation, large and small. With time the large colonies are able to produce cells expressing both hepatocyte and ductal markers. Furthermore, large colonies can be expanded extensively and produce functional hepatocytes *in vivo*. ECFC express the surface marker CD133 and are negative for CD45 (panhematopoietic marker) and Ter119 (red blood cell marker). Approximately one per 100 CD133+CD45−Ter119− cells from a DDC-treated liver form large epithelial colonies. Interestingly, cells with a similar surface phenotype from normal liver formed only small, but not large colonies.

ECFC have also been found in human liver specimens, and surface markers have been identified that can enrich for colony-forming cells. In human liver, only cells positive for EpCAM and CD133 are capable of forming colonies, and once again two distinct colony morphologies were observed. The large type1 colonies can be passaged and expanded significantly, suggesting self-renewal ability. Some *in vivo* hepatocytic differentiation was seen after transplantation of very large numbers of *ex vivo* expanded ECFC. It remains unclear whether the freshly-isolated cells also can produce hepatocytes without prior growth in culture. Importantly, at most one per 1000 EpCAM-positive cells formed colonies, indicating that only a small subfraction actually have progenitor activity. Thus, EpCAM or CD133 staining cannot be used to definitively localize human hepatic progenitors *in vivo*. Most bile duct epithelial cells are positive for these markers, and it is unlikely that all of them are progenitors. Data from both the murine and human systems currently indicate that the hepatic progenitors are a subset of the ductal cell population.

Extrahepatic Liver Cell Progenitors

Multipotent stem/progenitor cells have been isolated from numerous sources, and in many cases hepatocytic differentiation has been reported. These are non-transformed primary cell types capable of extensive cell division, while retaining the ability to be differentiated into hepatocytes *in vitro*. These cells are unlikely to be natural liver stem cells which are used in the organism, but nonetheless are of therapeutic and biomedical interest. In most of these systems only *in vitro* differentiation into cells resembling hepatocytes has been achieved, while *in vivo* assays indicate that the cells generated are not fully mature. I will therefore call them "hepatocyte-like."

Multipotent Adult Progenitor Cells and Mesenchymal Stem Cells as Hepatocyte Progenitors

Multipotent adult progenitor cells (MAPC) are a unique population of adult stem cells which can be isolated from the marrow of multiple mammalian species including human, rat, and mouse. MAPC are generated in culture by plating non-hematopoietic adherent cells and serial passaging. They are telomerase positive, and grow stably in culture for many passages if kept at low density. It remains unclear whether MAPC represent a population of cells that exists *in vivo* or whether their pluripotency represents spontaneous dedifferentiation in culture. These cells have been reported to have properties similar to embryonic stem cells, in that they can be differentiated towards many different lineages *in vitro* under the appropriate conditions. A protocol was developed which permitted the efficient induction of hepatocyte gene expression in human and murine MAPC. These conditions included culture on Matrigel, and use of fibroblast growth factor 4 (FGF-4) and hepatocyte growth factor (HGF). Over a two-week course, the majority of MAPC changed their phenotype and expressed multiple hepatocyte functions, including urea synthesis, albumin secretion, phenobarbital inducible cytochrome p450 induction, and others.

Like MAPC, mesenchymal stem cells (MSC) are usually bone marrow-derived and can be isolated by expanding the fraction of bone marrow cells that attaches to plastic tissue culture dishes. While they are clearly multipotent in terms of being able to yield multiple mesenchymal lineages *in vitro*, numerous reports indicate that they may also be differentiated into hepatocyte-like cells *in vitro*. The differentiation protocols sometimes involve treatment of the MSC with a demethylating drug, such as 5-azocytidine, followed by exposure to medium containing typical factors for hepatocytic differentiation, such as EGF and HGF. MSC derived from human fat have also been induced into a hepatocyte-like state characterized by albumin and glycogen production, as well as the ability to detoxify ammonia. Differentiation was induced over the course of three weeks in culture using multiple factors including dexamethasone, EGF, HGF, FGF1, FGF4, and oncostatin M. While MSC-derived hepatocyte-like cells have many characteristics of mature liver cells and can engraft *in vivo*, successful functional liver repopulation has not been observed.

Amniotic Stem Cells

Recent work from several laboratories has suggested that amniotic fluid and amniotic membranes contain pluripotent cells that can differentiated into multiple lineages, including endoderm. Specific protocols for the induction

of the hepatic phenotype have been developed and are similar to the differentiation regimens used for other progenitors. The hepatocyte-like cells generated with the most optimized regimens, express numerous cytochrome p450 enzymes involved in drug metabolism, as well as other markers typical of hepatocytes.

Embryonic Stem Cells

Much excitement has been generated by the notion that embryonic stem cells can serve as infinitely expandable precursors to hepatocytes. Clearly, both murine and human ES cells in culture cells can be induced along the endodermal and hepatocytic lineages by the sequential use of specific cytokines and growth factors. The prevailing paradigm suggests that natural embryonic development should be mimicked in order to obtain the most functional mature cells. Therefore, the first step of many protocols typically involves the generation of embryoid bodies, followed by the induction of definitive endoderm using activin A. The endodermal population can then be further specified towards the hepatic lineage using BMP-4 and basic FGF. Cells produced in this fashion express typical markers, such as albumin, but usually also express alpha-protein. They can then be transplanted into the liver with differentiation into both mature hepatocytes and bile duct epithelial cells. The level of liver repopulation obtained with hepatocyte-differentiated ES cells is very low, but is somewhat higher when the cells are transplanted into MUP-uPA/SCID mice. To date, most published ES differentiation protocols generate "hepatocyte-like" cells, but not fully-functional, mature, and transplantable equivalents of hepatocytes isolated from adult liver. However, it appears possible to select for more mature hepatocyte-like cells using surface markers, such as the asialoglycocoprotein receptor for purification. In the future, it appears likely that conditions will be developed in which lineage-specified ES cells will be therapeutically effective.

IMMORTAL LIVER PROGENITOR CELL LINES

Rat Cell Lines

WB-344 Cells

This cell line was clonogenically derived from non-parenchymal rat liver cells, and is probably the most intensely studied liver stem cell line. WB3-44 cells are likely derived from Canal of Hering cells. While WB-344 cells can be cultured indefinitely *in vitro*, they retain the ability to differentiate into morphologically normal hepatocytes after transplantation without forming tumors. To date, WB-344 cells are the only cells to fulfill this stringent criterion to represent a true liver stem cell line. Nonetheless, little is known about the molecular mechanisms regulating the stem cell to hepatocyte transition, and liver repopulation with this cell line has not yet been reported.

Rat Oval Cell Lines

Multiple laboratories have isolated oval cell lines from carcinogen-treated rats. Consistent with the proposed role of oval cells in the formation of hepatocarcinoma, these cell lines form tumors on transplantation into immune-deficient recipients. The isolation, culture, and transplantation of these cells have been well-reviewed.

Mouse Cell Lines

While it is normally difficult to establish permanent epithelial cell lines from mouse liver, such lines can be established routinely from transgenic mice which overexpress a constitutively-active form of the HGF receptor c-met. Two morphologically distinct types of cells emerge from such cultures. Both grow extensively in culture under certain media conditions, but can be induced to differentiate with the appropriate signals. Clonal cell lines with the "epithelial" morphology resemble hepatocytes, and give rise only to hepatocyte-like offspring. In contrast, "palmate" clones can give rise to two distinct lineages, depending on the differentiation conditions used. Some general conditions which can cause differentiation of palmate cells in either direction have been discovered. Acidic FGF or DMSO induces hepatocytic differentiation, whereas culture in Matrigel induced the formation of bile duct-like structures. Recently, culture conditions were developed to derive clonally-growing bipotential cell lines from normal embryonic mice, thus circumventing the need for c-met overexpression. These bipotential murine embryonic lines (BMEL) are not only capable of generating both hepatocytes and bile ducts *in vitro*, but also *in vivo*.

Another important line is HBC-3 cells, which are bipotential and derived from day 9.5 mouse embryonic liver. This clonal cell line can be induced to differentiate into hepatocytes by DMSO or sodium butyrate. HBC-3 has been useful in dissecting signals which govern lineage decisions.

Pig Cell Lines

An interesting cell line was established from cultured pig epiblast. PICM-19 cells are bipotential, similar to the murine cell lines reported above. In contrast to other cell lines, however, PICM-19 cells were derived from a very early embryo prior to the formation of a liver premordium.

Human Cell Lines

Only a single human cell line, AKN-1, which may have liver progenitor characteristics has been described.

Pancreas-derived Cell Lines

Several laboratories have produced cell lines from the adult pancreas and used them in transdifferentiation studies. In one study permanent cell lines with epithelial characteristics were established from both liver and pancreas under similar culture conditions. Extensive phenotypic characterization was performed, and under the conditions of analysis none of the cell lines expressed differentiated markers of either pancreas or liver. However, their morphological characteristics and expression profiles for aldolase, lactate dehydrogenase, and cytokeratins were indistinguishable, suggesting that primitive epithelial cells from both organs were very similar.

Others also established permanent "duct" epithelial cell lines from the rat, which expressed cytokeratins 8 and 19 and carbonic anhydrase. These cells were then implanted in a collagen matrix into normal rat, either subcutaneously or into the peritoneal cavity. On transplantation, several hepatocyte-specific markers were expressed, and pancreatic duct markers were silenced. This was particularly true for those cells transplanted into the mesenteric fat pad. The expression levels of albumin, transferrin, and aldolase B approached those seen in normal adult hepatocytes. Again, these data are consistent with the hypothesis of a common endodermal hepato-pancreatic stem cell.

Transplantable Liver Repopulating Cells

One way to define stem cells is by their ability to repopulate the respective organ and restore its function. The hematopoietic stem cell, for example, was defined by reconstitution of the blood lineages of lethally-irradiated hosts. In the 1990s, similar repopulation assays were developed for the liver in several animal models. In liver repopulation a small number of transplanted donor cells engrafts in the liver, the cells expand and replace >50% of the liver mass. Thus, it has now become possible to perform experiments analogous to those done in the hematopoietic system, including cell sorting, competitive repopulation, serial transplantation, and retroviral marking. Hepatic stem cells can now be defined functionally by their ability to repopulate liver. It should be emphasized that liver repopulation refers to replacement of only the hepatocytes by transplanted cells. Efficient repopulation of the biliary system by transplanted cells has not yet been reported.

The main animal models for liver repopulation studies are summarized in Table 34-2. In all cases, liver repopulation by transplanted cells is based on a powerful selective advantage for the transplanted cells over the pre-existing host hepatocytes. In many models, this selection is achieved by genetic differences (transgene/knockout), but DNA damage has also been used successfully, particularly in the rat. Selective gamma-irradiation of the liver holds particular promise as a method which may have clinical translational potential.

The animals described above have been utilized to determine the nature of transplantable liver repopulating cells, and to determine whether undifferentiated stem cells are driving this process. The stem cell hypothesis was strengthened by the observation that liver repopulating cells could be serially transplanted for >100 cell doublings without loss of functionality. Interestingly, the only donor-derived cells in this experiment were hepatocytes. No biliary epithelium or other cell types of donor origin were found, thus raising the possibility of a "unipotential" stem cell.

It has also become possible to achieve liver repopulation with human hepatocytes. Immune-deficient mice have been crossed with strains which provide a selective environment, including albumin-uPA transgenics, Fah-deficiency, and some chemical treatment regimens. These

TABLE 34-2 Animal Models for Liver Repopulation

Animal	Human cell repopulation	Model	Selective pressure
Mouse	yes	Albumin-urokinase transgenic	Urokinase mediated hepatocyte injury
Mouse	yes	Fah knockout	Accumulation of toxic tyrosine metabolite
Mouse		Albumin-HSVTK transgenic	HSVTK mediated conversion of gancyclovir to toxin
Mouse		Mdr3 knockout	Bile acid accumulation
Mouse		Bcl2- transgenic donor cells	fas ligand (Jo2) induces apoptosis in hepatocytes not expressing Bcl2
Rat/mouse	yes	Retrorsine conditioning	Host hepatocytes are inactivated by retrorsine (DNA damage)
Rat		Conditioning by radiation	Host hepatocytes are inactivated by X-rays (DNA damage)

models will serve as *in vivo* assays for human hepatocyte progenitors in the future.

Hepatocytes as Liver Repopulating Cells

It is reasonable to hypothesize that adult liver cells are not homogeneous in their capacity for cell division, and that sub-populations with high repopulation capacity might exist. In the hematopoietic system, repopulation experiments with purified fractions of total bone marrow were used to identify sub-populations with high reconstitution activity. Similar experiments have recently been performed with liver cells and indicate that, in contrast to the hematopoietic system, differentiated hepatocytes themselves have a high capacity for liver repopulation.

In the *Fah* mutant mouse model, size fractionation, retroviral marking, and competitive repopulation between naïve liver cells and those that had been serially transplanted were performed. All three experimental approaches indicated that large, binucleated hepatocytes, representing ~70% of the population, were primarily responsible for liver repopulation. Others confirmed this finding by transplanting hepatocytes which had been sorted based on their ploidy (DNA content). No differences in the repopulating ability of 2n, 4n, and 8n hepatocytes were found. Thus, no evidence for a rare stem cell responsible for liver repopulation was detected. Together, these experiments strongly suggest that fully-differentiated hepatocytes, which constitute the majority of liver cells, are efficient in liver repopulation and have a stem cell-like capacity for cell division.

Hepatocytes appear to not be strictly unipotential, at least *in vitro*. Mature rat hepatocytes can differentiate into ductular epithelium; and *in vivo* transplantation experiments are also consistent with ductular differentiation *in vivo*. These results are provocative, in that they suggest the possibility that there are no stem cells in the liver at all, but that all regenerative responses, including the oval cell response, rely on hepatocytes.

It is important to note that the nature of the human cell capable of liver repopulation has not yet been definitively determined. However, the large size of clonal revertant nodules ($>2\,\text{cm} = 2 \times 10^8$ cells) in the livers of human patients with hereditary tyrosinemia, indicate that mature human hepatocytes have extensive regenerative potential.

Liver Repopulation by Non-hepatocytes

Despite the evidence that hepatocytes themselves are serially transplantable liver repopulating cells, other cell types are also capable of repopulating the liver. This finding is analogous to the situation in liver regeneration where both hepatocytes themselves, as well as undifferentiated hepatocyte progenitors, are capable of reconstituting the organ. In the following text, liver repopulation by several non-hepatocyte cell types will be described: (1) Liver repopulation by fetal hepatoblasts; (2) oval cells; (3) pancreatic liver progenitors; (4) hematopoietic stem cells; and (5) embryonic stem cells.

Liver Repopulation with Fetal Hepatoblasts

During embryonic development, the fetal liver bud contains hepatoblasts, cells which express α-fetoprotein, as well as hepatocyte (albumin) and biliary (CK19) markers. These cells, therefore, may represent fetal liver stem cells capable of hepatocyte repopulation, and potentially also reconstitution of the biliary system.

Several reports on transplantation of hepatoblasts have been published. One study using fetal rat liver cells in the retrorsine model indicated that there were at least three distinct sub-populations of hepatoblasts at ED 12–14. One population appeared to be bipotential, on the basis of histochemical markers, and the other two had either a unipotent hepatocyte or a biliary epithelial cell phenotype. After transplantation, the bipotential cells were able to proliferate in retrorsine-treated cell transplantation recipients, whereas the unipotent cells grew even in untreated rats. However, none of the fetal liver cell populations proliferated spontaneously. Partial hepatectomy or thyroid hormone treatment was required to augment proliferation of transplanted cells. Nonetheless, fetal liver cells proliferated more readily than adult cells. Finally, the transplanted fetal cells gave rise to both hepatocyte cords and mature bile duct structures. It was not formally proven, however, that both of these cell lineages originated from a clonal precursor. Cell sorting has now been applied to identify the cell surface characteristics of murine and rat liver repopulating hepatoblasts. In day 12.5 embryos the liver-repopulating cells were E-cadherin, Dlk1, and Sca1 positive, but negative for other hematopoietic markers, such as c-kit, CD34, and CD45. In the rat, the repopulating activity also resided with Dlk-1 positive cells. Interestingly, only cells negative for Thy-1 were able to repopulate, thus shedding doubt on the utility of this marker to isolate hepatoblasts or oval cells. Liver repopulation has also been achieved with human hepatoblasts, confirming the results obtained with rodent cells. Together these results indicated that transplanted fetal hepatoblasts proliferate more readily than adult hepatocytes, and some fetal liver cells may remain bipotential.

Liver Repopulation by Oval Cells

Oval cells are similar to fetal hepatoblasts, in that they are also bipotential. These cells have therefore been of interest in liver repopulation experiments. Transplantation of either hepatic- or pancreatic-derived oval cells has been reported in the rat. Upon transplantation, the oval cells proliferated modestly and differentiated into mature hepatocytes, even under non-selective conditions. However, because no *in vivo* selection model was used, the true capacity for

liver repopulation was not demonstrated in these experiments. Recently, however, repopulation studies have been performed with purified murine oval cells, induced by DDC. These experiments clearly indicated that oval cells have extensive liver repopulation capacity and constitute a potential population for use in cell therapy. Bipotential progenitor colonies derived from the livers of DDC-treated mice and expanded significantly *in vivo* are also capable of repopulation *in vivo*.

Liver Repopulation with Pancreatic Progenitors

During embryogenesis, the main pancreatic cell types develop from a common endodermal precursor located in the ventral foregut, including ducts, ductules, acinar cells, and the endocrine α, β, and δ cells. Importantly, the main epithelial cells of the liver, hepatocytes, and bile-duct epithelium (BDE) are also thought to arise from the same region of the foregut endoderm. This tight relationship between liver and pancreas in embryonic development has raised the possibility that a common hepato-pancreatic precursor/stem cell may continue to persist in adult life in both the liver and pancreas. Indeed, several independent lines of evidence suggest that adult pancreas contains cells which can give rise to hepatocytes. The earliest description of pancreatic hepatocytes was in hamsters treated with a pancreatic carcinogen, N-nitroso-bis(2-oxopropyl)amine (BOP) in 1981. Similarly, small, occasional clusters of hepatocyte-like cells were also found in rats being treated with a peroxisome proliferator, Wy-14643. Interestingly, some of the same carcinogens can be used to produce hepatic oval cells, particularly when combined with partial hepatectomy. The best known example is the emergence of hepatocytes in copper-depleted rats after re-feeding of copper. In this system, weanling rats are fed a copper-free diet for eight weeks, which leads to complete acinar atrophy, and then are re-fed copper. Within weeks, cells with multiple hepatocellular characteristics emerge from the remaining pancreatic ducts. This work has been interpreted to suggest the presence of a pancreatic liver stem cell. This notion is also supported by the appearance of hepatocellular markers in human pancreatic cancers. More recently, a specific cytokine has been identified as a candidate to drive this process. Transgenic mice, in which the keratinocyte growth factor (KGF) gene is driven by insulin promoter, consistently develop pancreatic hepatocytes. Thus, the existence of pancreatic liver precursors has been shown in several different mammalian species, and under multiple experimental conditions. There is also evidence for the reverse, i.e., the existence of pancreatic precursors in the liver. Some cases of liver tumors, particularly cholangiocarcinomas, display expression of markers typical for pancreatic cell types. In one small series of liver tumors analyzed, 61% of cholangiocarcinomas expressed pancreatic type amylase. Other tumors have been reported to produce lipase, another enzyme specific for pancreatic exocrine cells. Similarly, cultured rat oval cell lines derived from liver could be differentiated *in vitro* into cells with regulated insulin secretion, i.e., properties similar to pancreatic β-cells. These cells were even able to rescue diabetic rats in transplantation experiments. Others have demonstrated the emergence of insulin secreting cells *in vivo* in the liver of animals treated with vectors expressing developmental pancreatic transcription factors. Therefore, one can hypothesize that both adult liver and adult pancreas continue to harbor a small population of primitive hepato-pancreatic stem cells, with the potential to give rise to the same differentiated progeny as during embryogenesis.

Transplantation experiments have been conducted to verify the pancreatic liver stem cell hypothesis. As mentioned above, pancreatic oval cells induced by copper depletion were shown to give rise to morphologically normal hepatocytes *in vivo*. In addition, the Fah knockout model has been used to demonstrate the existence of hepatocyte progenitors in adult pancreas. The pancreata of mice wild-type for *Fah* were digested into single cells, and then transplanted into *Fah* mutant mice. Importantly, the cells used in these experiments were from normal adult pancreata, and no chemical treatments or oval cell induction regimens were used in the donor mice. Although only ~10% of pancreas cell transplanted *Fah* mutant mice survived long-term and had extensive liver repopulation, >50% of recipients had donor derived Fah+ hepatocyte nodules. Based on the number of hepatocyte nodules formed, it could be estimated that pancreatic liver precursors were rare (~1/5000) in the donor cell population. Additional experiments showed that the pancreatic ducts themselves did not give rise to hepatocytes in the liver repopulation assay. These findings are not inconsistent with the results obtained with the copper depletion model, because both periductular interstitial cells, as well as the ducts themselves, were considered candidates to be oval cell precursors.

A hypothetical model for the lineage relationships between hepatic and pancreatic cells is illustrated in Figure 34-4. This model predicts the existence of an adult hepato-pancreatic stem cell, similar to the ventral foregut endodermal precursor which exists during embryonic development. Formal proof of this model has not yet been achieved.

Liver Repopulation With Bone Marrow-derived Progenitors

Recent work has documented that the adult bone marrow of mammals contains cells with a variety of differentiation capacities. The hematopoietic stem cell (HSC) giving rise to all blood cell lineages has been known to reside in this compartment for many years. However, bone marrow also contains mesenchymal stem cells (MSC) capable of differentiating into chondrocytes, osteoblasts, and other connective tissue cell types. While HSC are non-adherent,

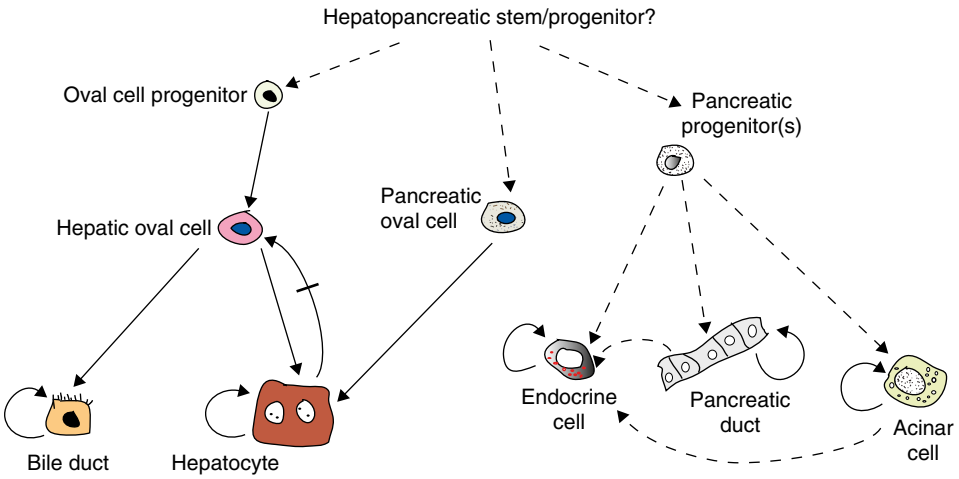


FIGURE 34-4 Progenitor lineage relationships in adult liver and pancreas. The thickness of the arrows indicates the dominant mode of regeneration. Stippled lines delineate rare or hypothetical cell fate transitions that occur only under specific experimental conditions.

MSC adhere to plastic dishes in tissue culture, and can be expanded there. Thus, adult bone marrow had been shown to produce a variety of tissue types of mesodermal origin. In 1998, Petersen *et al.* first suggested that bone marrow also contained epithelial precursors. Bone marrow and whole liver transplantation between genetically-distinct animals were used to demonstrate that a portion of liver oval cells were donor-derived after induction with 2-acetylaminofluorene. Theise *et al.* showed that bone marrow-derived hepatocytes also exist in the mouse, and that oval cell induction was not required for this phenomenon. Other reports demonstrated that donor-derived epithelial cells are also present in human patients which have undergone a gender-mismatched bone marrow transplantation. In all these studies, the epithelial nature of the cells was demonstrated morphologically, and by the expression of hepatocyte specific markers.

Hematopoietic Stem Cells as Hepatocyte Precursors

Cell sorting experiments and transplantation of purified hematopoietic stem cells of the KTLS phenotype (c-kit(hi)Thy(lo)Lin−Sca-1+) and at limiting dilution showed that the primitive HSC was the bone marrow cell responsible for the donor-derived hepatocytes in *Fah* knockout mice. This study also demonstrated extensive liver repopulation, and a complete correction of the metabolic liver disease hereditary tyrosinemia, thus demonstrating conclusively that bone marrow-derived hepatocytes function normally.

It also has been suggested that human HSC, particularly those isolated from cord blood, can also give rise to hepatocyte-like cells in a xenotransplantion setting. Human CD34+ cord blood cells were engrafted into NOD–SCID mice after low dose irradiation using the standard procedure. If such animals were then exposed to liver injury, particularly carbon tetrachloride, human cells which produce albumin, a hepatocyte marker can be found at low frequency (<1/1000). This finding suggests that human HSC may also be able to produce hepatocytes, especially after injury. This finding has been reported independently by multiple groups. It has been suggested that only a subpopulation of HSC, positive for receptor for the complement molecule C1q (C1qR(p)) is capable of giving rise to hepatocytes. However, because of the rarity of the human albumin producing cells, it is unknown whether these cord blood-derived cells only express some hepatocyte markers, or whether they are true hepatocytes with all requisite functions.

Physiologic Significance of Bone Marrow Derived Hepatocytes

Despite the unambiguous finding that fully-functional bone marrow-derived hepatocytes really exist, there has been considerable controversy regarding their functional importance in liver injury. In one model, illustrated in Figure 34-5A, replenishment of liver cells from the bone marrow is an important injury-response pathway, particularly in progenitor dependent regeneration (oval cell response). Hepatocytes would be generated either by direct differentiation of HSC, or indirectly via an oval cell intermediate. The opposing model, illustrated in Figure 34-5B, suggests that oval cell precursors reside only in the liver itself (tissue resident stem cell), and that the bone marrow contributes very few liver epithelial cells, even during injury. To determine whether liver damage enhances the transition of HSC to hepatocytes, the degree of cell replacement after BMT in mice has been measured not only in healthy animals, but also in the context of pre-existing hepatocyte injury or oval cell regeneration. Importantly, the frequency of bone marrow-derived hepatocytes was not higher in the acute liver injury seen in Fah knockout mice than in healthy control livers. In addition, no contribution from bone marrow precursors to the oval cell reaction was

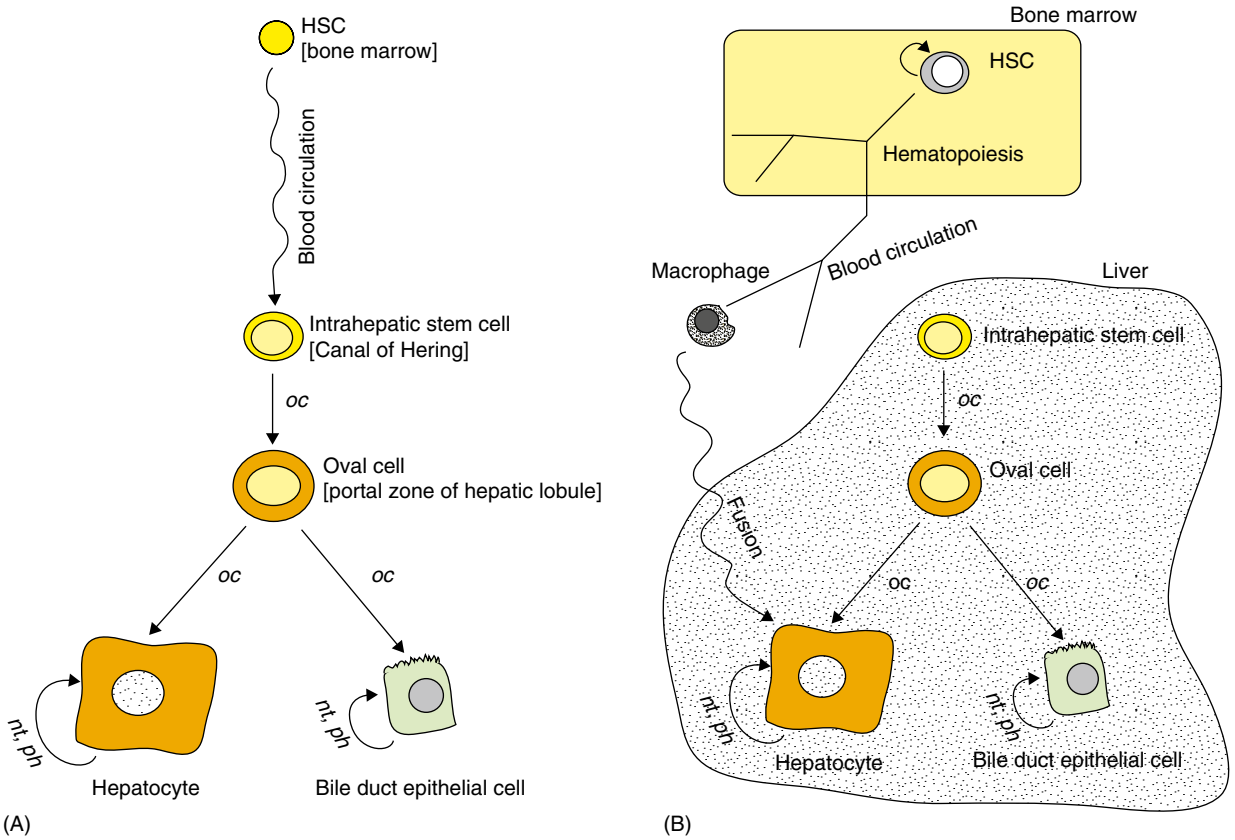


FIGURE 34-5 Models for the role of bone marrow resident stem cells in liver cell maintenance. The different kinds of liver regeneration (see text) are indicated: nt = normal tissue turnover, ph = regeneration after partial hepatectomy, oc = progenitor dependent regeneration (oval cell response). (A) Transdifferentiation model. The anatomic location of the cells is given in square brackets. Hematopoietic stem cells (HSC) give rise to hepatocytes by trafficking to the liver via the blood circulation to then engraft and differentiate into epithelial cell precursors (intrahepatic stem cells). These cells can become activated during progenitor-dependent liver regeneration (oval cell response) and give rise to oval cells. Oval cells differentiate into hepatocytes or bile ducts. (B) Fusion model. There is no direct lineage relationship between HSC and liver epithelial cells. Cell fusion between circulating progeny (macrophages) of the HSC and hepatocytes are responsible for the emergence of bone marrow derived liver epithelial cells. Progenitor dependent liver regeneration (oc) occurs by activation of intrahepatic liver stem cells, which are not derived from the bone marrow.

induced by the DDC. Together, these results indicate that HSC do not serve as epithelial cell precursors in all forms of hepatic injury. The ability of MSC to acquire hepatocytic properties *in vitro* has recently suggested the possibility that MSC, not HSC are the hepatocyte precursors.

Mechanism for the Formation of Bone Marrow Stem-derived Hepatocytes

Three basic mechanisms for the repopulation of liver by bone marrow derived cells can be considered. First, bone marrow could theoretically harbor a specialized endodermal stem cell capable of producing hepatocytes and other epithelial cells. This cell would be analogous to the mesenchymal stem cell which produces mesodermal derivatives such as muscle, cartilage, and fat. Second, hepatocytes and blood cells could be derived from the same stem cell by hierarchical differentiation (Figure 34-5A, transdifferentiation model). This was initially the hypothesis favored by many in the field. Third, bone marrow derived hepatocytes could potentially be derived not from differentiation at all, but rather by cell fusion (Figure 34-5B, fusion model). This possibility was raised by the observation that hematopoietic cells could spontaneously fuse with embryonic stem cell *in vitro*, and then produce multiple tissues in chimeric mouse embryos.

In order to address these hypotheses, transplantation experiments were designed in which genetic markers for both donor marrow and host hepatocytes were used in the *Fah* knockout model. The results from this study were unambiguous: the vast majority of bone marrow-derived hepatocytes contained genetic information from both the donor and host, indicating cell fusion. Cytogenetic analysis of female to male gender mismatched transplant indicated a high frequency of tetraploid XXXY and hexaploid XXXXYY karyotypes, predicted for fusion. Importantly, similar experiments were independently performed by another laboratory, and confirmed that fusion was the predominant mechanism for derivation of hepatocytes from

bone marrow stem cells in the *Fah* knockout model. It is now clear that myelomonocytic cells, and not HSC themselves, are responsible for the bulk of hepatic fusion events.

KEY WORDS

Bone marrow-derived hepatocyte An engrafted hepatocyte with a chromosomal or expression marker that identifies it as possessing genetic material from a (usually transplanted) bone marrow cell; can be the product of a hematopoietic cell/hepatocyte fusion event, differentiation by a hematopoietic progenitor with hepatic lineage potential or differentiation by a hepatic-committed marrow-resident progenitor.

Liver stem cell A poorly characterized cell proposed as the precursor of oval cells, or to comprise the most primitive subset of oval cells; the term is also used to refer broadly to all liver progenitor cells.

Oval cell A small cell with an oval-shaped nucleus and a high nuclear/cytoplasmic size ratio that emerges in the portal zone under conditions of chronic liver damage; can differentiate into the hepatocyte or bile duct epithelial lineages and thus is a bipotential liver progenitor.

Pancreatic liver stem cell A cell located in the pancreas that can generate hepatocytes to support liver repair upon transplantation; may be a bipotential hepato-pancreatic precursor or an extrahepatic progenitor committed to hepatic differentiation.

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Chapter 35

Pancreatic Stem Cells

Yuval Dor and Douglas A. Melton

INTRODUCTION

From a clinical perspective, the pancreas is an important focus of stem cell research, because it is an attractive target for cell replacement therapy. In type I diabetes, the insulin-producing B-cells that reside in the pancreatic islets of Langerhans are destroyed by autoimmune attack, and it is thought that self-renewing stem cells could provide an unlimited source of B-cells for transplantation. Such therapeutic efforts require the prospective isolation of stem cells, with the potential to produce B-cells and the development of methods to direct their expansion and differentiation.

From a developmental biology perspective, the role of stem cells in the pancreas is a fascinating problem. New cells are produced during adulthood, but their origin is not clear. Much of the field is focused on the identification of B-cell progenitors and the characterization of their molecular requirements, but it is not known what role such cells play during pancreas maintenance and regeneration, or whether the adult pancreas contains a population of true stem cells. Regardless, information about the specification of the B-cell fate from undifferentiated progenitors will be important in directing the differentiation of stem cells *in vitro.* In this chapter, we review evidence for the existence and identity of pancreatic progenitor and stem cells and describe the criteria for experimental demonstration of such cells.

DEFINITION OF STEM CELLS AND OF PROGENITOR CELLS

The most rigorous definition of a stem cell is a cell that, upon proliferation, produces some progeny that have the same developmental potential (a process called self-renewal), as well as other progeny that have a more restricted developmental potential. Such cells may be present transiently during embryonic development, or persistently during the entire life of the organism, but their defining property is self-renewal. A progenitor cell, on the other hand, is any cell that generates another differentiated cell type.

Another important notion in stem cell biology, with potential relevance to the pancreas, is that of the facultative stem cell. These cells are thought to be functional, differentiated cells in an adult organ that can dedifferentiate in response to a specific signal (usually believed to be tissue damage), and then differentiate into another cell type. For example, the liver is thought to contain facultative stem cells that reside in the bile ducts and are capable of producing hepatocytes. Thus, the bile duct cells provide an effective reservoir of liver progenitors in case of tissue damage, although they may not meet the criteria of being stem cells at the single-cell level.

How can we demonstrate that a particular cell is a stem cell? Formally, such a demonstration must be based on clonal analysis: a single cell has to be followed over time in order to show that it can produce more stem cells, as well as differentiated cells. The gold standard for the identification of stem cells was set in the hematopoietic system, where clonal analysis has been carried out *in vitro* (by subcloning individual colonies), as well as *in vivo* (by serial transplantation of single stem cells into lethally irradiated mice). By contrast, the identification of progenitor cells is an easier task. It requires demonstration, using some sort of lineage-tracing experiment, that an undifferentiated cell population generates differentiated cells.

Even when stem cells cannot be identified or isolated in a particular organ, their existence may be inferred from kinetic studies of 5′-bromo-2′-deoxyuridine (BrdU) incorporation. Because stem cells are believed to be slowly-dividing, the presence of label-retaining cells can point to the anatomical location of a stem cell niche. Such an analysis was carried out in self-renewing organ systems, such as skin and hair. In light of these definitions, in the next sections we discuss the evidence for stem cells and progenitors during pancreas development and adult life.

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PROGENITOR CELLS DURING EMBRYONIC DEVELOPMENT OF THE PANCREAS

The adult pancreas contains three major cell types: exocrine cells, organized in acini, which secrete digestive enzymes; duct epithelial cells which flush these enzymes to the duodenum; and endocrine cells, organized in the islets of Langerhans, that secrete hormones to the blood. The islets, accounting for ~1% of the cells in the adult pancreas, contain four major cell types that secrete different hormones: A, B, D, and PP-cells secreting glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. In recent years, a detailed cellular and molecular understanding of pancreas development has emerged (Figure 35-1). Although many questions remain, one recurring theme is the essential role played by progenitor cells in organogenesis. Early patterning of the gut tube generates a sheet of epithelial cells that express the homeobox gene *pdx1* around embryonic day 9 (E9) in the mouse. These epithelial cells bud from the gut tube, proliferate, and branch to form a tubular structure around E12.5, from which cells differentiate and organize into the exocrine and endocrine tissues of the mature pancreas. Numerous experiments demonstrate that this process is based on the proliferation and step-wise differentiation of progenitor cells. As shown by lineage-tracing experiments using tamoxifen-induced Cre recombinase, the early *pdx1*+ cells (which by E10.5 also express the transcription factor p48/ptf1) produce all cell types in the adult pancreas. Later on, progenitors for duct, acinar, and endocrine lineages segregate, proliferate, and differentiate. Endocrine progenitors migrate from the tubular structure of the early pancreas, and coalesce to form the islets of Langerhans just before birth. During that period, they undergo further restrictions in their potential, marked by the transient expression of several genes. Most notably, neurogenin3 expression marks progenitor cells for all endocrine lineages. Down-regulation of progenitor markers and expression of the hormone genes mark the terminal differentiation of endocrine cells. A similar mechanism of gradual commitment, though less well-defined, is thought to act in the developing exocrine pancreas.

How do pancreatic progenitors choose their fates? A long-standing hypothesis is that the axis of mitosis in epithelial cells of the early pancreas correlates with, or even determines, the identity of daughter cells (Figure 35-1). When the plane of division is perpendicular to the lumen, both daughter cells remain epithelial (symmetric division), and may lobulate to form exocrine acini. When the plane of division is parallel to the lumen, one daughter cell detaches from the lumen, and may become an endocrine progenitor cell (asymmetric division). This hypothesis, although untested, supports the popular view that adult pancreatic stem cells reside in the ducts. More recently, genetic evidence has suggested that cell–cell interactions mediated by the Notch pathway affect lateral specification of progenitor cells at multiple developmental

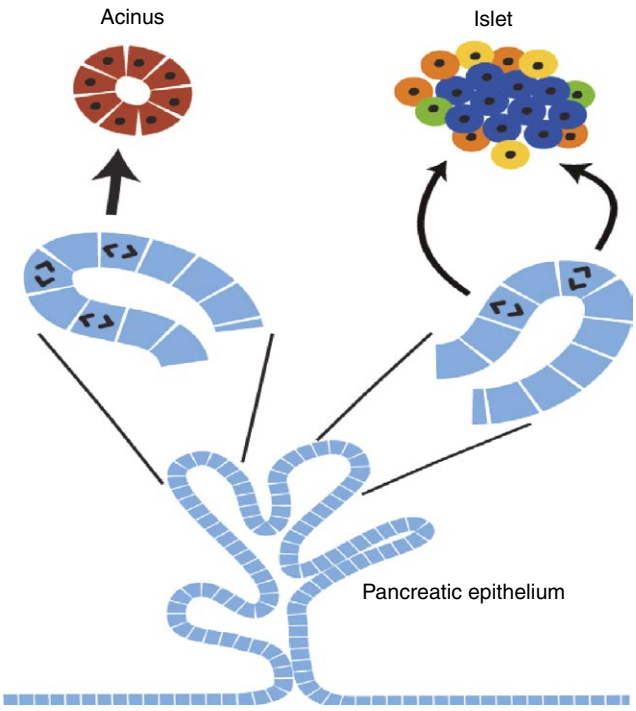


FIGURE 35-1 Embryonic development of the pancreas. Pdx1+ epithelial cells of the early pancreas (pale blue); acinar cells (red); and A-, B-, D-, and PP-cells (indicated by orange, blue, yellow, and green in the islet). The proposed plane of division of endocrine and exocrine progenitors is indicated.

junctions. Accordingly, mutations in Notch pathway genes can lead to accelerated, premature endocrine differentiation at the expense of the exocrine pancreas. These two models for specification may be compatible, as the Notch pathway is known to affect the plane of division in neural progenitors.

What is the relative contribution of progenitors and differentiated cells to proliferation of the embryonic pancreas? Although this question has not been addressed directly, the fate of hormone-expressing cells that appear early in pancreas development (E9.5–10.5) was recently examined. These cells, many of which coexpress multiple endocrine hormones, were initially thought to replicate and generate the mature islets. However, lineage-tracing and ablation experiments suggest that the multiple-hormone-expressing cells do not contribute to the adult endocrine pancreas. Rather, mature endocrine cells appear to be derived from midgestation progenitors.

Following this progenitor-based formative stage, rapid growth of the pancreas in late gestation and early postnatal life is thought to involve gradually less differentiation of progenitor cells, and more replication of fully-differentiated cells (see later sections of this chapter).

Although the identity and importance of progenitors during pancreas development is clear, there is no indication for true self-renewal of cells during this period. The recent demonstration of heterogeneity among the Pdxl+ cells of the early pancreas suggests that lineage segregation occurs very

early during pancreas organogenesis. Furthermore, no clonal analysis of the embryonic pancreas has been carried out *in vivo*. *In vitro* clonal analysis demonstrated the common origin of the exocrine and endocrine pancreas, but not self-renewal. Therefore, it is fair to conclude that progenitor cells play a major role in pancreas formation. However, there is no evidence for self-renewing pancreatic stem cells during embryonic development. We now turn to the postnatal growth and maintenance of the pancreas, with special attention to B-cells. Do progenitor cells of the embryonic type persist in adult life in an active or latent form? Is there evidence for stem cells capable of generating new pancreatic cells in the adult?

PROGENITOR CELLS IN THE ADULT PANCREAS

When describing stem/progenitor cells in the adult pancreas, it is important to deal separately with two questions: first, what is the turnover rate of the different components of the pancreas during postnatal growth, throughout adult maintenance, and in response to injury? Second, are the new cells derived from stem/progenitor cells (neogenesis), or from replicating differentiated cells? It must be remembered that an impressive capacity for homeostatic maintenance, or even regeneration, does not indicate neogenesis. For example, dramatic regeneration of the injured liver can occur solely by proliferation of differentiated hepatocytes, without a requirement for stem cells.

Evidence from Cell Dynamics

After birth, the growth of the pancreas continues, but slows significantly around one month of age in the mouse and rat. However, even in old animals there is a measurable rate of cell birth in all pancreatic compartments. In the B-cell compartment, where most studies have been done, the replication rate falls from ~5% in four-week-old animals to ~0.1% in mice older than three months. Embryonic-type progenitors (based on expression patterns) are not seen in the normal adult animal (with the possible exception of rare islet cells generated from neurogenin3+ progenitors).

In spite of its low basal turnover rate, significant hyperplasia is seen in the adult pancreas under certain physiological and pathological conditions. For example, during pregnancy the B-cell mass doubles, a response attributed to a combination of cell hypertrophy and cell proliferation. More dramatically, several reports have documented an ability of the B-cell compartment to recover from genetically-programmed, autoimmune, surgical, or chemical damage.

Can the new cells, in normal homeostasis or in a regeneration setting, be fully accounted for by the replication of differentiated cells? Empirically, for the B-cell compartment, can the number of BrdU+ pulse-labeled B-cells explain the accumulation of BrdU+ B-cells following continuous administration of BrdU? If not, the presence of undifferentiated progenitors must be invoked. This type of analysis has, however, proven difficult because it requires reliable values for several elusive parameters: what is the duration of the S phase and the total cell cycle in a specific compartment? What is the death rate, and how long does it take for a dying cell to be cleared? Furthermore, cell number is not easily inferred from total cell area as obtained by immunostaining, because of cellular hypertrophy. Thus, a study of B-cell dynamics must include B-cell counting by fluorescence-activated cell sorting or by careful histological analysis across the whole pancreas, a criterion that is not always met.

The most comprehensive effort in this direction was carried out by Finegood *et al.* (1995) who studied B-cell dynamics throughout the lifespan of the rat. Their results imply a significant contribution of progenitor cells to the B-cell mass in the first weeks after birth, and then a shift to tissue maintenance by slow replication of B-cells. In addition, significant B-cell neogenesis was deduced in a similar study of chronic hyperglycemia in rats. These kinetic studies suggest that adult pancreatic progenitors exist. However, they do not help determine the molecular and anatomical origins of these cells.

Another argument for the existence of B-cell progenitors is the identification of single B-cells embedded in the adult exocrine tissue. These isolated cells are reportedly more frequent following insults, which led to the notion that they are newly-generated from progenitors that reside in ducts or acini (see later sections of this chapter). The new B-cells are then believed to coalesce into islets in a mechanism resembling developmental islet morphogenesis. However, careful analysis is required to distinguish this interpretation from other potential explanations (e.g., disintegration of existing islets). In fact, recent genetic lineage tracing experiments suggest that the small clusters of B-cells are derived from B-cells, rather than from stem/progenitor cells.

A combination of BrdU pulse-chase experiments and genetic labeling of differentiated cells (for example, using an inducible Crerecombinase) may allow a direct comparison between the contribution of progenitors and the contribution of differentiated cells to pancreas growth and maintenance.

Many experiments neglect the kinetic aspects of neogenesis, and focus on histological identification of progenitors, based on expression markers in certain anatomical locations. However, without lineage tracing, these studies cannot demonstrate the fate of the putative progenitors, or determine their importance. In the next sections, we describe the most notable proposals regarding the identity of adult pancreatic stem-progenitor cells.

Ducts

It is widely believed that adult pancreatic stem/progenitor cells are located in the duct epithelium. Indeed, cells expressing B-cell markers (insulin, glut-2, pax6, isl1, and

HNF3b) can often be found embedded in or adjacent to adult ducts following an insult to the pancreas.

Moreover, duct cell replication (as assessed by BrdU incorporation) is increased after such insults. In addition, adult duct preparations are claimed to be capable of endocrine differentiation *in vitro* (see later sections of this chapter). Conceptually, the appearance of endocrine cells in or near ducts is interpreted as recapitulation of embryonic pancreas morphogenesis, where endocrine progenitors bud from the epithelium. However, adult ducts are not necessarily identical in their genetic program to the embryonic tubes that should be referred to as "duct-like structures." Indeed, recent lineage experiments indicate that the definitive ductal and endocrine lineages are separated as early as E12.5. With regard to endocrine cells seen budding from ducts, caution is required when suggesting dynamic interpretations to static histological snapshots (Figures 35-2 and 35-3).

The preceding description shows the importance of specific duct markers for the assessment of the ductal origins of endocrine cells. Although several duct markers have been found (such as carbonic anhydrase, cystic fibrosis transmembrane-conductance regulator, and cytokeratin-19), so far none has been translated to a useful lineage marker in transgenic mice.

In summary, it seems that ducts can elicit a proliferative response to tissue damage, and that endocrine markers are occasionally expressed in cells of the ductal epithelium. However, the fate of these cells and their relative contribution to the endocrine or exocrine pancreas has yet to be demonstrated.

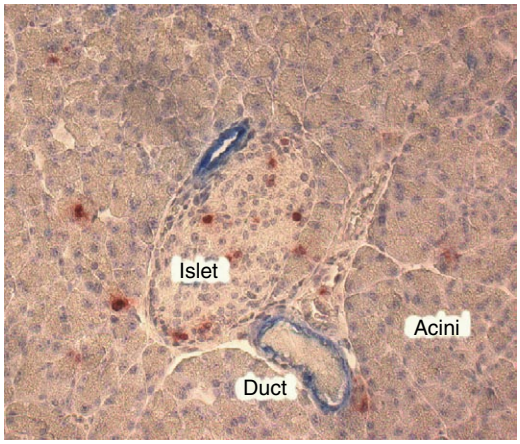


FIGURE 35-2 Histology of adult pancreas. Exocrine tissue (acini and ducts) and endocrine tissue (islet of Langerhans) are shown. Section from a four-week-old mouse pancreas, pulse labeled with BrdU (red). Ducts are stained blue. Note the close association between the ducts and the islet. Original magnification = 200 ×.

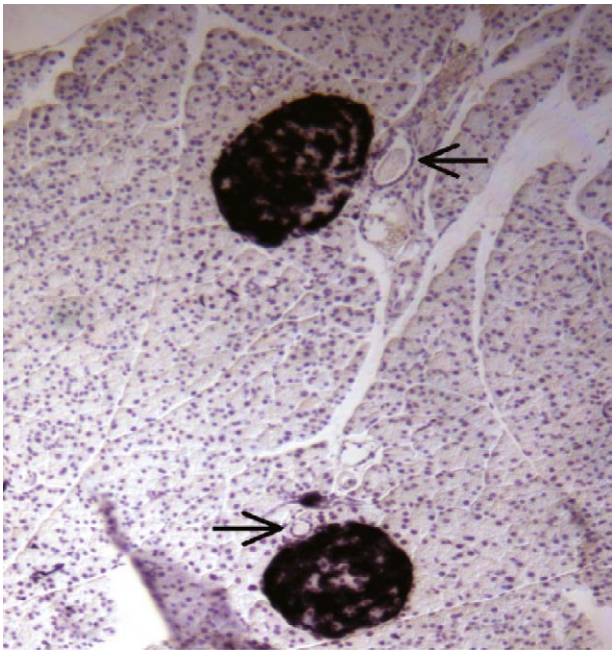


FIGURE 35-3 Lineage labeling of the endocrine pancreas. Section from the pancreas of a four-week-old ngn3-cre; Z/AP double-transgenic mouse, stained for alkaline phosphatase activity (dark blue). In this mouse, cre-mediated recombination in *ngn3*+ embryonic endocrine progenitor cells leads to heritable expression of the human placental alkaline phosphatase gene. Only islet cells are labeled, suggesting that ducts (arrows) and acini never expressed the *ngn3* gene. Original magnification = 100 ×.

Acini

Starting from the observation of isolated B-cells embedded in exocrine tissue, other investigators have proposed that acinar cells can transdifferentiate, with or without replication, into endocrine cells. This possibility is supported by *in vitro* studies showing that an acinar cell line can adopt endocrine features under certain conditions. However, as in the case of duct-embedded progenitors, the lineage of these cells was not followed *in vivo*; therefore, their origin and fate could not be determined. In this case, however, good lineage markers exist, so the possible acinar origin of endocrine cells can be directly tested. Preliminary experiments with an Elastase promoter-driven Cre recombinase have suggested that, under normal conditions, Elastase-expressing cells do not produce endocrine or duct cells. It remains to be tested, however, whether acinar cells contain "facultative" endocrine progenitors activated upon injury.

Intraislet Progenitors

Several groups have suggested the existence of intraislet progenitor cells capable of proliferation and differentiation into B-cells. These reports are based on the expression of putative stem-progenitor cell markers in islets. One such proposal is that the expression of Nestin, a marker of neuronal progenitors, labels intraislet endocrine progenitors with a potential for *in vitro* differentiation into several fates. However, a recent lineage analysis using Cre recombinase driven by the Nestin

promoter showed that pancreatic endocrine cells do not form by the differentiation of Nestin+ cells. In addition, Nestin was recently shown to be expressed in the mesenchymal, but not in epithelial, cells of the embryonic pancreas, further questioning its relevance for pancreatic lineages.

Others have documented the coexpression of pancreatic hormones in islets following a diabetogenic insult. For example, It was proposed that following streptozotocin treatment, somatostatin+ cells and glucagon+ cells generate B-cells by proliferation and differentiation through somatostatin+Pdxl+, and glucagon+glut-2+ intermediate cell types. These proposals have yet to be confirmed by lineage analysis.

Finally, clinical transplantation of islets may provide important clues about islet cell dynamics. Islet grafts into the portal vein (as done with diabetic patients), or under the kidney capsule (as done routinely with rodents), survive for many months and provide glycemic control. Although it is clear that cells in the grafts die and proliferate, it is not known if new endocrine cells in the graft are derived only from replicating differentiated cells or also from progenitors. Since purified islets with minimal exocrine tissue are used for transplantations, any indication for the existence of progenitors in the graft would point to an intraislet source.

Bone Marrow

A recent study found a significant contribution of bone marrow-derived cells to the B-cell compartment in the absence of tissue damage other than irradiation. To facilitate the detection of marrow-derived B-cells, marrow was taken from transgenic mice expressing Cre recombinase under the insulin promoter, as well as enhanced green fluorescent protein (eGFP), preceded by a Cre-removable transcriptional stop signal. Thus, bone marrow cells of the donor mice should express eGFP only if they turn on the insulin promoter. By sorting eGFP+ cells in pancreatic islets of recipient mice, up to 3% of all B-cells were identified from a donor origin two months after marrow transplantation. The elegant design of the experiment was such that the fusion of marrow cells with recipient B-cells could be ruled out. These surprising results still await independent confirmation.

Other Evidence for Adult Pancreatic Progenitor Cells

It has been reported that cells in the adult pancreas can reconstitute a degenerating liver deficient in the enzyme fumarylacetoacetate hydrolase. This observation suggested the existence of progenitors in the pancreas capable of liver differentiation. However, more recent results indicate that liver reconstitution by bone marrow cells represents complementation of defective hepatocytes by cell fusion, rather than transdifferentiation of marrow cells to liver. Thus, it is likely that liver reconstitution by pancreatic cells represents the "fusibility" rather than the plasticity of these cells.

In summary, indirect evidence suggests that endocrine progenitor cells exist, or can appear, in the adult pancreas following injury. Careful lineage analysis has yet to demonstrate the existence, origin, fate, and importance of these cells to pancreas homeostasis. Similar to the situation in the embryo, there is no evidence for the existence of self-renewing stem cells in the adult pancreas.

Very recently, we have used a genetic lineage analysis system to study the dynamics of adult pancreatic B-cells. This analysis shows that adult B-cells are maintained largely by self-duplication, rather than stem cell differentiation during normal life in the mouse, as well as during regeneration from pancreatectomy. The results cast doubt on the idea that stem cells contribute significantly to the formation of new B-cells during adult life. We cannot rule out the existence of adult pancreatic stem cells; however, it seems that the contribution of such putative cells to new B-cells is minimal. In addition, facultative pancreatic stem cells might exist and be recruited following other types of injury. The same system can now be used for testing claims about stem cell differentiation into B-cells, *in vitro* and *in vitro.*

FORCING OTHER TISSUES TO ADOPT A PANCREATIC PHENOTYPE

From a therapeutic perspective, artificial induction of a B-cell fate in other cell types is as useful as finding and expanding endogenous pancreatic progenitors. Moreover, nonpancreatic cells, if indeed competent to adopt a B-cell program, may be more accessible for expansion and *in vitro* manipulation. Efforts in this direction have focused on tissues thought to be capable of pancreatic differentiation because of a common lineage origin or a similar genetic program. Here, we explain the pancreatic connection of the most popular starting tissues for such experiments.

Liver to Pancreas

Several observations led to the notion that the adult liver may be a close relative of the pancreas in terms of the steps required for switching fates. First, both organs are derived from the endoderm of the embryonic gut tube. Moreover, studies by Zaret and colleagues suggest that a bipotential liver–pancreas progenitor cell population exists by E8 in the ventral foregut endoderm. Using embryo tissue explantation experiments, they have shown that fibroblast growth factor signaling from the cardiac mesoderm can divert these progenitors from a default pancreatic fate to a liver fate. However, lineage or clonal analysis was not performed to formally demonstrate that bipotential cells exist.

Second, transdifferentiation of pancreas to liver, in which clusters of hepatocytes emerge in the pancreas, occurs

spontaneously in humans and can be induced experimentally in rats. In cultured pancreatic cells, liver transdifferentiation was demonstrated by activation of a single transcription factor (C/EBP-b) without a requirement for cell division. These observations suggest that adult liver and pancreas cells may retain a degree of plasticity that allows transdifferentiation to occur. Alternatively, a rare stem-progenitor cell population in these organs, yet to be identified, may be responsible for the metaplastic phenomenon.

Notably, no evidence has been found for deep similarities in the genetic program of the adult pancreas and liver. Based on these observations, several groups have tried experimentally to convert liver to pancreas by enforced expression of key pancreatic genes. One candidate for imposing a pancreatic fate is Pdxl, the most upstream component of the pancreatic program. Indeed, adenoviral-mediated transfer of Pdxl to adult mice was reported to induce low-level expression of insulin or exocrine pancreas markers in the liver.

A conceptual problem with simple overexpression of Pdxl is the need for specific transcriptional cofactors to be present. For example, Pdxl is known to require the Pbx and Meis proteins for proper function. With the hope of bypassing the need for Pdxl cofactors absent from the liver, a Pdxl-VP16 fusion gene was recently constructed. Transgenic expression of Pdxl-VP16 (but not unmodified pdxl) in livers of *Xenopus* tadpoles led to significant expression of pancreatic endocrine and exocrine genes in the liver, concomitant with down-regulation of liver genes. The use of a liver-specific promoter (from the transthyretin gene), and the finding that the same construct induces a pancreatic phenotype in a hepatocyte cell line, suggests that the responding cells are differentiated hepatocytes rather than uncommitted progenitors or stem cells.

In summary, it seems that ectopic expression of pancreatic transcription factors in the embryonic and perhaps the adult liver can lead to activation of a pancreatic program. The available data suggest that the mechanism is direct hepatocyte transdifferentiation, rather than activation of a common liver–pancreas progenitor. In addition to the therapeutic potential of the approach, analysis of the molecular details may provide significant insights into the problems of competence and potential.

Neurons to Pancreas

Although arising from different lineages during embryogenesis, the endocrine pancreas and the nervous system share a significant portion of their developmental programs. For example, most transcription factors participating in the step-wise differentiation of the endocrine pancreas are also expressed in the developing brain. Some examples are isl1, Hb9, neurogenin3, NeuroD, Nkx2.2, Nkx6.1, pax6, and brn4. Notable exceptions are the absence of Pdxl, as well as insulin, expression in the brain. However, the insulin promoter does contain neuronal regulatory elements that are repressed in the context of the endogenous insulin locus, but give rise to neural expression when driving transgenes. The significance of this intriguing similarity is not fully understood. One possibility is that it reflects a common ancestral lineage for brain and endocrine pancreas, which diverged during evolution as the endocrine program came under Pdxl control. This idea is supported by the finding that insulin and glucagon are expressed in specific neurons in the fly (which lacks a pancreas). Thus, neuronal progenitors are the closest known relatives of pancreatic progenitors in terms of their genetic program. This suggests that neuronal progenitors (easily derived from ES cells) may be only a few steps from adopting a pancreatic fate. However, attempts to induce ES-derived neural progenitors to a pancreatic fate have so far failed.

Gut to Pancreas

Several recent studies explored the potential of the nonpancreatic segments of the digestive tract to adopt a pancreatic fate. Ectopic expression of Pdxl in the embryonic gut outside the normal expression domain of Pdxl led to a partial adoption of a pancreatic fate, judged by gene expression and cell budding out of the epithelium. Similarly, ectopic expression of neurogenin3 in the embryonic chick or mouse led to the appearance of endocrine cells expressing glucagon and somatostatin (but not insulin). Inactivation of ptfla-p48, a transcription factor normally expressed in pancreatic progenitors, led to a cell-autonomous switch in the reverse direction, namely, the conversion of pancreas to duodenal epithelium. These results suggest that, even after the onset of segment-specific genetic programs, the embryonic gut epithelium remains plastic. It is not known if such plasticity is retained in the adult intestine.

IN VITRO STUDIES

Numerous attempts have been made to generate B-cells *in vitro* from dissociated pancreatic tissue using the available information on the identity of putative pancreatic stem-progenitors. Accordingly, pancreatic duct preparations were cultured, and the appearance of differentiated cell types was monitored. Some groups have reported on the generation of endocrine cells from duct cultures, and some even claimed to correct hyperglycemia in animals by grafting the *in vitro*-generated islet-like clusters. Others have used islet preparations as a starting material. These studies, however, share several methodological problems that complicate their interpretation. First, the number of endocrine cells at the beginning and at the end of the culture period was not always measured carefully. Second, all studies were carried out on enriched rather than pure cell populations, thus not ruling out the presence of contaminating cells from unintended compartments. Third, clonal analysis

was not performed. Fourth, many of these studies used insulin immunostaining as a marker for B-cell differentiation, and supplied large amounts of exogenous insulin in the culture medium. Therefore, they may have scored for insulin uptake rather than *de novo* synthesis.

Perhaps a more promising approach to the problem is the use of embryonic pancreas as a starting material, since it is clear that it contains progenitor cells that can proliferate and differentiate. This, however, has also proved a difficult task. Much of the difficulty may result from the lack of proper culture conditions that mimic the embryonic environment.

SUMMARY

The search for pancreatic stem or progenitor cells may provide insights into the basic mechanisms of organ homeostasis, as well as a promising therapeutic approach for diabetes. It is clear that pancreatic progenitor cells exist and play a major role during embryonic development, although much has to be learned about the mechanisms and molecules controlling them. There is no evidence, however, for pancreatic stem cells capable of self-renewal.

As for the adult pancreas, no conclusive evidence shows the importance or mere existence of pancreatic progenitor or stem cells in the adult organism. On the contrary, it appears that, at least for pancreatic beta cells, the major mechanism for tissue maintenance is self-duplication rather than stem cell differentiation. It is possible, however, that under certain stresses progenitor cells appear and may contribute to the maintenance of the pancreas. Most evidence points to the pancreatic ducts as the likely niche of such "facultative" progenitors. Development of culture conditions that support the growth and differentiation of dissociated pancreatic tissue, as well as *in vivo* lineage analysis systems, are the tools most needed for tackling this problem.

Other cell types may retain a degree of plasticity that allows for pancreatic differentiation under artificial conditions. Embryonic stem cells are the only cells available *in vitro* that can definitely differentiate into pancreatic cell types; however, this potential has yet to be demonstrated *in vitro*. Theoretical considerations and some experimental evidence suggest that neuronal progenitors, liver cells, and intestinal epithelial cells may also be forced to adopt a pancreatic fate.

KEY WORDS

Genetic lineage tracing The use of permanent labeling, usually DNA recombination, to mark a defined cellular population and trace its progeny.

Neurogenin3 A transcription factor expressed in endocrine progenitor cells of the embryonic pancreas.

Pdx1 Pancreatic and duodenal homeobox gene-1; a transcription factor essential for pancreas development. During embryogenesis, Pdx1 is expressed in stem/progenitor cells, giving rise to all cell types in the adult pancreas. During postnatal life, its expression becomes restricted to beta cells.

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Chapter 36

Stem Cells in the Gastrointestinal Tract

Stuart A. C. McDonald, Trevor A. Graham, Adam Humphries, Nicholas A. Wright, Sean L. Preston, Mairi Brittan, and Natalie C. Direkze

Turnover of the epithelial cell lineages within the gastrointestinal tract is a constant process, occurring every two to seven days under normal homeostasis and increasing after damage. This process is regulated by multipotent stem cells, which generate all gastrointestinal epithelial cell lineages and can regenerate whole intestinal crypts and gastric glands. The stem cells of the gastrointestinal tract are as yet undefined, although it is generally agreed that they are located within a "niche" in the intestinal crypts and gastric glands. Studies of allophenic, tetraparental chimeric mice and targeted stem cell mutations suggest that a single stem cell undergoes an asymmetrical division to produce an identical daughter cell, thus replicating itself, and a committed progenitor cell, which further differentiates into an adult epithelial cell type. The discovery of stem cell plasticity in many tissues, including the ability of transplanted bone marrow to transdifferentiate into intestinal subepithelial myofibroblasts, provides a potential use of bone marrow cells to deliver therapeutic genes to damaged tissues, for example, in the treatment of mesenchymal diseases in the gastrointestinal tract, such as fibrosis and Crohn's disease. Studies are beginning to identify the molecular pathways that regulate stem cell proliferation and differentiation into adult gastrointestinal cell lineages, such as the Wnt and Notch–Delta signaling pathways, and to discover the importance of mesenchymal–epithelial interactions in normal gastrointestinal epithelium and in development and disease. Finally, despite some dispute, a strong case can be made for intestinal neoplasia arising as a result of a series of mutations in stem cells. The mechanism and direction of the spread of this mutated clone in the gastrointestinal mucosa is hotly disputed, and central to this argument is the position and nature of the gastrointestinal stem cell.

INTRODUCTION

There has been a tremendous increase in interest in stem cell biology and its potential applications in recent years. Although this has been galvanized by the exploitation of research in embryonic stem cells, it is interesting to note that what might be called the "intestinal stem cell community," albeit small, has been working productively for some 40 to 50 years. With stem cells now claiming considerable attention, in retrospect, many of the basic tenets that govern our understanding of organ-specific stem cells have come from studies in the gastrointestinal tract and the haematopoietic system.

In the gastrointestinal tract, there is a large body of evidence that multipotential stem cells are found in specific zones, or niches, within gastric glands and intestinal crypts, composed of and maintained by myofibroblasts in the adjacent lamina propria. In this chapter, we review evidence that these multipotential stem cells generate all gastrointestinal epithelial cell lineages through committed precursor cells housed in the proliferative compartments of intestinal crypts and gastric glands, a concept that has had a long and difficult gestation. Notwithstanding their obvious significance, the gastrointestinal stem cells remain elusive and unidentified, mainly because of a lack of accepted morphological and functional markers at the single-cell level. We also explore concepts of stem cell number, location, and fate, and we touch on the ability of gastrointestinal stem cells to regenerate cell lineages of whole intestinal crypts and villi after damage. The luminal gut shows regional specializations of function—the stomach primarily for absorption and the intestinal mucosa for both absorption and secretion. This is reflected by variation in the adult cell lineages native to each tissue, and it is thereby consistent that stem cell fate within each tissue is also different. We are beginning to understand the mechanisms that govern such variation. Controversial findings regarding stem cell plasticity in the gastrointestinal tract are examined in this chapter. Because of their longevity, putatively the same as that of the organism itself, stem cells are often viewed as the target cells for carcinogens, and the cells of origin for spontaneous tumors. This includes a "top-down" proliferation of mutated stem cells located within intercryptal zones on the mucosal surface, which spread downwards into the adjacent crypts. Contrasting to this is a "bottom-up" theory of the upward proliferation of

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mutated stem cells in the crypt base, to produce dysplastic crypts that replicate and expand by crypt fission. This brings other facets of gut biology into sharp focus—the mechanisms of crypt reproduction; the clonal architecture of normal and dysplastic gastric glands, intestinal crypts, and their derivative tumors; and the role that stem cells take in these events. Here, we propose that the stem cells accumulate the multiple genetic events leading to tumorigenesis, and we explore the manner by which such mutated clones spread in gastrointestinal epithelia. Many of these concepts are being explored at the level of molecular regulatory pathways, including the signaling pathways of Wnt and transforming growth factor β (TGFβ).

This is quite a brief, since several of the most common tumors originate in the gastrointestinal tract, and with increasing incidence in Barrett's esophagus. Our concepts of stem cell biology impinge considerably on our understanding of how these tumors arise.

GASTROINTESTINAL MUCOSA CONTAINS MULTIPLE LINEAGES

In the small intestine, the epithelial lining forms numerous crypts and larger, finger-shaped projections called villi. In the colon there are many crypts, which vary in size throughout the colon; the shortest is in the ascending colon. Overall, four main epithelial cell lineages exist in the intestinal epithelium. These are the columnar cells, the mucin-secreting cells, the endocrine cells, and Paneth cells in the small intestine. Other less-common cell lineages are also present, such as the caveolated cells, and membranous or microfold cells. Columnar cells, with apical microvilli, are the most abundant epithelial cells, termed enterocytes in the small intestine and colonocytes in the large intestine. "Goblet" cells, containing mucin granules—and thus producing swollen, goblet-shaped cells—are found throughout the colonic epithelium, secreting mucus into the intestinal lumen. Endocrine, "neuroendocrine," or "enteroendocrine" cells form an abundant cell population distributed throughout the intestinal epithelium; these cells secrete peptide hormones in an endocrine or paracrine manner from their contained dense core of neurosecretory granules. Paneth cells are located almost exclusively at the crypt base of the small intestine and ascending colon, they contain large apical secretory granules, and express several proteins—including lysozyme, tumor necrosis factor, and the antibacterial cryptins (low molecular-weight peptides related to defensins).

In the stomach, the epithelial lining forms long, tubular glands divided into foveolus, isthmus, neck, and base regions. Gastric foveolar or surface mucus cells are located on the mucosal surface and in the foveola. They contain tightly-packed mucous granules in the supranuclear cytoplasm, and do not possess a theca. The mucus neck cells are situated within the neck and isthmus of the gastric glands, and contain apical secretory mucin granules. The peptic–chief or zymogenic cells are located in the base of the glands in the fundic and body regions; they secrete pepsinogen from oval zymogenic granules. The parietal or oxyntic, acid-secreting cells are located in the body of the stomach in the base of the glands. These cells have many surface infoldings, or canaliculi, which form a network reaching almost to the base of the gland. Endocrine cell families include the enterochromaffin-like cells in the fundus or body that produce histamine; the gastrin-producing cells are a major component of the antral mucosa.

The intestinal crypts and gastric glands are enclosed within a fenestrated sheath of intestinal subepithelial myofibroblasts (ISEMFs). These cells exist as a syncytium that extends throughout the lamina propria and merges with the pericytes of the blood vessels. The ISEMFs are closely applied to the intestinal epithelium, and play a vital role in epithelial–mesenchymal interactions. ISEMFs secrete hepatocyte growth factor (HGF), TGFβ, and keratinocyte growth factor (KGF), but the receptors for these growth factors are located on the epithelial cells. Thus, the ISEMFs are essential for the regulation of epithelial cell differentiation through the secretion of these and possibly other growth factors. Platelet-derived growth factor-α (PDGF-A), expressed in the intestinal epithelium, acts by paracrine signaling through its mesenchymal receptor, PDGFR-α, to regulate epithelial–mesenchymal interactions during development. Studies of mice with targeted deletions in the PDGF-A or PDGFR-α genes have shown defects in normal proliferation and differentiation of PDGFR-α-positive mesenchymal cells. Typically, ISEMFs are α-smooth muscle actin-positive (αSMA+) and desmin−, but some myofibroblasts also express myosin-heavy chains. ISEMFs undergo proliferation despite this MyoD expression—unlike the skeletal muscle myoblast, which decycles once MyoD is expressed. It has been proposed that these cells form a renewing population, migrating upwards as they accompany the epithelial escalator. Although they appear to proliferate and migrate, they move relatively slowly, and then move off into the lamina propria to become polyploid.

A second myofibroblast population in the intestine is the interstitial cells of Cajal. These cells are located close to neurones in the muscular layers; they act as pacemakers for gastrointestinal smooth muscle activity, propagate electrical events, and modulate neurotransmission. They are said to be αSMA+ and desmin+, and to immunostain for *c-kit* and CD45.

EPITHELIAL CELL LINEAGES ORIGINATE FROM A COMMON PRECURSOR CELL

Little is known of the location and fate of the stem cells within the gastrointestinal tract, because of the lack of

distinctive and accepted stem cells markers, although they are usually said to appear undifferentiated and can be identified operationally by their ability to repopulate crypts and glands after damage.

The Unitarian hypothesis states that all the differentiated cell lineages within the gastrointestinal epithelium emanate from a common stem cell origin. Although widely propounded, little definitive evidence exists to underpin this hypothesis. Moreover, Pearse and coworkers famously proposed that gastrointestinal endocrine cells derive from migrating neuroendocrine stem cells in the neural crest, a concept that still has its adherents. Although studies of quail neural crest cells transplanted into chick embryos, or experiments where the neural crest is eradicated, show gut endocrine cells to be of endodermal origin, Pearse subsequently suggested that the endoderm is colonized by "neuroendocrine-programmed stem cells" from the primitive epiblast, which generate gut endocrine cells. This hypothesis was not ruled out by chick–quail chimera experiments; therefore, other models must be used to ascertain the gut endocrine cell origins, such as the chimeric mouse studies described later.

Several lines of evidence suggest that stem cells reside in the base of the crypts of Lieberkuhn in the small intestine, just superior to the Paneth cells (approximately the fourth or fifth cell position in mice). In the large intestine, they are presumed to be located in the midcrypt of the ascending colon, and in the crypt base of the descending colon. However, within the gastric glands, migration of cells is bidirectional from the neck–isthmus region to form the simple mucous epithelium of the foveolus or pit, and cells migrate downwards to form parietal cells and chief cells. Therefore, the stem cells are believed to be within the neck–isthmus region of the gastric gland. The Unitarian hypothesis is now supported by a considerable body of research.

SINGLE INTESTINAL STEM CELLS REGENERATE WHOLE CRYPTS CONTAINING ALL EPITHELIAL LINEAGES

The ability of intestinal stem cells to regenerate epithelial cell populations of entire intestinal crypts and villi following cytotoxic treatment has been demonstrated using the crypt microcolony assay. Four days after irradiation, sterilized crypts undergo apoptosis and disappear, but they can be identified by remaining radio-resistant Paneth cells at the crypt base. At higher radiation dose levels, only single cells survive in each crypt, since a unit increase in radiation leads to a unit reduction in crypt survival. Survival of one or more clonogenic cells in a crypt after radiation ensures crypt persistence, and there is regeneration of all epithelial cell populations of that crypt and, in the small intestine, of the overlying villi. Therefore, following cytotoxic damage, a single surviving stem cell can produce all cell types of the intestinal epithelium to reproduce a crypt.

MOUSE AGGREGATION CHIMERAS SHOW THAT INTESTINAL CRYPTS ARE CLONAL POPULATIONS

Mouse embryo aggregation chimeras are readily made, wherein the two populations can be easily distinguished. The lectin *Dolichos biflorus* agglutinin (DBA) binds to sites on the B6-derived, but not on the SWR-derived, cells in C57BL/6J Lac (B6)↔SWR mouse embryo aggregation chimeras, and it can be used to distinguish the two parental strains in gut epithelium. The intestinal crypts in each chimera studied were either positive or negative for DBA, and there were no mixed crypts in the tens of thousands studied. Therefore, each crypt forms a clonal population. This is the case for Paneth, mucous, and columnar cells, although it was not possible to detect the markers in endocrine cells, because of their inability to bind the lectin on their surface. In neonatal C57BL/6J Lac (B6)↔SWR chimeras, there were mixed (i.e., polyclonal) crypts for the first two weeks after birth, suggesting that multiple stem cells exist during development. However, all crypts ultimately become derived from a single stem cell between birth and postnatal day 14, so-called monoclonal conversion. This apparent cleansing or "purification" of crypts could be caused by the stochastic loss of one stem cell lineage, or by the segregation of lineages because of an extremely active replication of crypts by fission, which occurs at this developmental period. To exclude the possibility that crypts from distinct strains segregate differentially during organogenesis, Griffiths confirmed the findings in mice bearing an X-linked defective gene for glucose-6-phosphate dehydrogenase (*G6PD*) (Figure 36-1A–C).

In the stomach, the situation is similar, although more complex. Epithelial cell lineages in the antral gastric mucosa of the mouse stomach, including the endocrine cells, derive from a common stem cell. Identification of the Y-chromosome by *in situ* hybridization in XX–XY chimeric mice showed that gastric glands were also clonal populations. These findings were confirmed by Tatematsu in 1994 in CH3↔BALB/c chimeric mice, where each gastric gland was composed of either CH3 or BALB/c cells; there were no mixed glands. Thus, we might advance the general hypothesis that gastric glands in the mouse, in addition to the intestinal crypts, are clonally derived. Additionally, by combining immunohistochemistry for gastrin, an endocrine cell marker, with *in situ* hybridization to detect the Y-chromosome, the male regions of the gastric glands were shown to be almost exclusively Y-chromosome positive with gastrin-positive endocrine cells, whereas the female areas in the chimeric stomach were gastrin positive and Y-chromosome negative. These results finally negate the

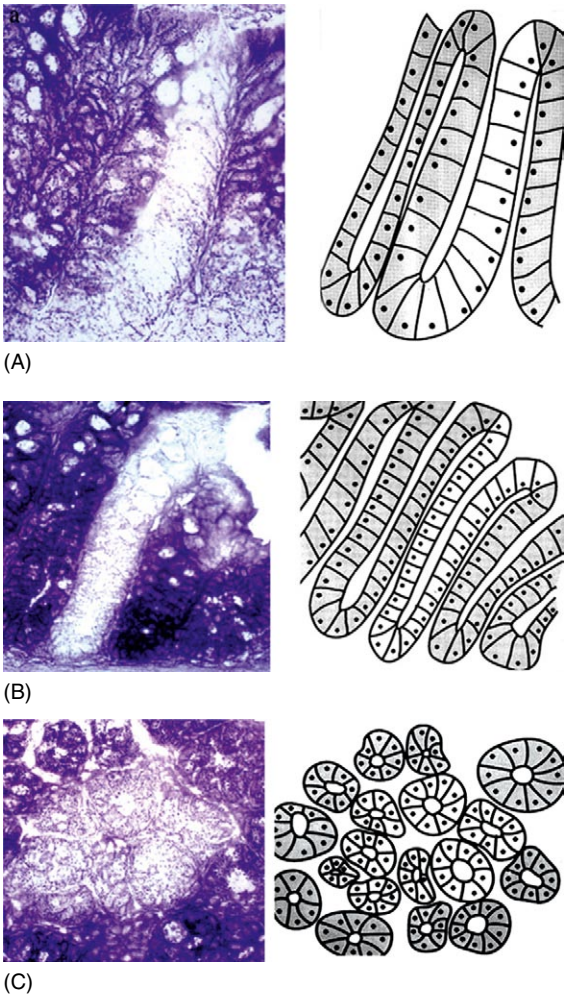


FIGURE 36-1 Gastrointestinal clonality studies. (A-C) G6PD histochemistry in frozen sections of colonic mucosa in an ENU-treated C3H mouse: (A) partially negative crypt, (B) completely negative crypt, and (C) cross section of an eight-crypt patch at 21 weeks. with permission from Park.

Pearse concept that gut endocrine cells originate from a separate stem cell pool.

Nomura used X-inactivation mosaic mice expressing a *lacZ* reporter gene to study clonality of gastric glands in the fundic and pyloric regions of the developing mouse stomach. As in the intestine, most glands are initially polyclonal, with three or four stem cells per gland, but they become monoclonal during the first six weeks of life—again either by purification of the glands, where division of one stem cell eventually overrides all other stem cells, or by gland fission. A population of approximately 5–10% of mixed, polyclonal glands persists into adulthood. The significance of these mixed glands with an increased stem cell number is not known, but it is possible that they do not undergo fission or have reduced fission rates; perhaps they even have an increased stem cell number during development, and therefore maintain a higher number of stem cells after crypt fission.

SOMATIC MUTATIONS IN STEM CELLS REVEAL STEM CELL HIERARCHY AND CLONAL SUCCESSION

Somatic mutations at certain loci allow us to study stem cell hierarchy and clonal succession within the gastrointestinal tract. Mutations in the *DLb-1* on chromosome 11 are one good example of this; C57BL/6J↔SWR F1 chimeric mice show heterozygous expression of a binding site on intestinal epithelial cells for the DBA lectin. This binding site can be abolished when the *Dlb-1* locus becomes mutated, either spontaneously or by the chemical mutagen ethyl nitrosourea (ENU). After ENU treatment, crypts emerge that initially are partially, and then entirely, negative for DBA staining. Perhaps the simplest explanation for this phenomenon is that a mutation occurs at the *Dlb-1* locus in a stem cell within the small intestinal crypt. This mutated cell could expand stochastically to produce a clone of cells that cannot bind DBA, and remain unstained. If this is the case, then a single stem cell can generate all the epithelial lineages within an intestinal crypt of the small intestine.

A "knockin" strategy at the *Dlb-1* locus can also be used to explain the preceding findings. If SWR mice do not express a DBA-binding site on their intestinal epithelial cells, but can be induced to bind DBA by ENU treatment, wholly DBA+ or DBA− intestinal crypts would result. From the use of this model, Bjerknes and Cheng propose that "committed epithelial progenitor" cells exist in mouse intestinal crypts by visualizing the morphology, location, and longevity of mutant clones in crypts and villi of the mouse small intestine. These transitory committed progenitor cells—the columnar cell progenitors (C_o) and the mucus cell progenitors (M_o)—evolve from pluripotential stem cells, and then differentiate further into adult intestinal epithelial cell types.

Not much is known about the mechanisms that regulate the proliferation of these progenitor cells, but administration of proglucagon-derived, glucagon-like peptide 2 (GLP-2) to SWR mice was found to induce intestinal epithelial growth and repair, specifically by stimulating the columnar cell progenitors, resulting in increased crypt and villus size in the normal small intestine. The receptor for GLP-2 (GLP2R) has been shown to be located on enteric neurons, and not on the gut enteroendocrine cells and in the brain as previously thought. GLP-2 activation of enteric neurons produces a rapid induction in *c-fos* expression, which signals growth of columnar epithelial cell progenitors and stem cells that generate adult columnar cell types. There is no stimulatory effect on the mucus cell lineage; instead, it is stimulated by KGF. Thus, the committed progenitor cells are involved in regeneration of damaged epithelia, possibly through a neural regulatory pathway (Figure 36-2B).

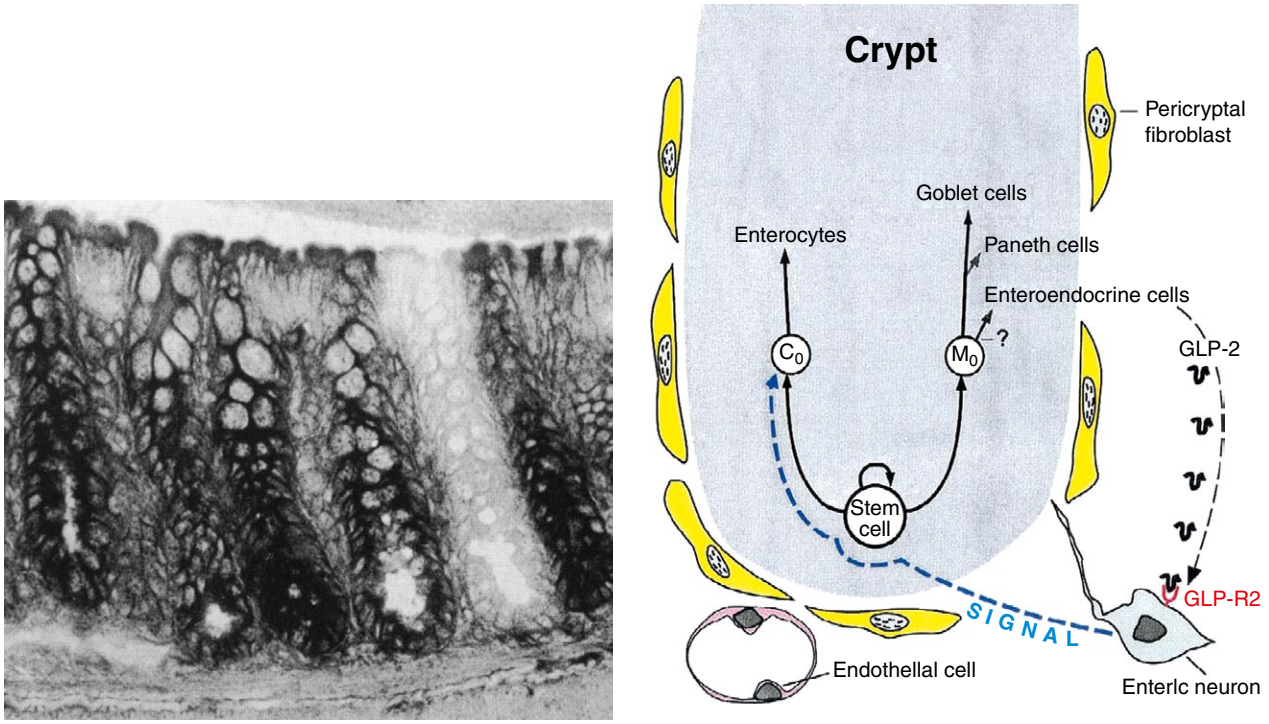


FIGURE 36-2 Stem cell niche hypothesis. (A) G6PD-stained frozen section of colonic mucosa from a CH3 mouse 14 days after a single dose of DMH, showing a single, wholly mutated crypt in which all cells have been replaced by a mutant phenotype. (B) Diagrammatic representation of the stem cell niche. An active, multipotent stem cell produces a daughter cell (C_o) generates enterocytic lineages and another that generates goblet, Paneth, and enteroendocrine cells (although it is not known whether all derive from M_o). GLP-2, produced by a subset of enteroendocrine cells, stimulates proliferation of the C_o daughter through an interaction with enteric nervous system neurons that express the GLP-2 receptor (GLP-2R). Panel A reproduced with permission from Williams and panel B reproduced with permission from Mills.

The possibility remains that cells from different parental strains of chimeric animals segregate independently during development to produce "monophenotypic" crypts and not "monoclonal" crypts, as we assumed earlier in this chapter. Mosaic expression of the electrophoretic isoenzymes PGK-1A and PGK-1B were analyzed in colonic crypts of mice heterozygous for the X-linked alleles *Pgk-1*^{a} and *Pgk-1*^{b}. In agreement with the results from the chimeric studies, no mixed crypts were seen, thus eliminating the possibility that these crypts are monophenotypic, and verifying that they are derived from a single progenitor cell. In a further model, mice that heterozygously express a defective *G6PD* gene have a crypt-restricted pattern of G6PD expression, thus confirming that the intestinal crypts are derived from a single stem cell. Moreover, mice treated with the colon carcinogen dimethylhydrazine (DMH) or ENU also develop crypts that initially are partially, and later are wholly, negative for G6PD. The partially-negative crypts could conceivably result from the mutation of a cell in the dividing transit population of the crypt that lacks stem cell properties. This is supported by the observation that these partially-negative crypts are transient and decrease in frequency, parallel to an increase in wholly-negative crypts. Conversely, such partially-negative crypts could become wholly-negative by stochastic expansion of a mutant stem cell. Wholly-negative crypts would then be a clonal population derived from this mutant stem cell.

After administration of a mutagen, in both the *Dlb-1* and the G6PD models, the time taken for the decrease in partially mutated crypts and the emergence of entirely negative crypts to reach a plateau is approximately four weeks in the small intestine, and up to 12 weeks in the large intestine (Figure 36-2A). This difference is intriguing, and was initially thought to be because of cell cycle time differences between the colon and the small intestine. However, a favored explanation can be found in the "stem cell niche hypothesis." This hypothesis suggests that multiple stem cells occupy a crypt with random cell loss after stem cell division, and was originally formulated as the stem cell zone hypothesis by Bjerknes and Cheng. The numbers of stem cells may be larger in the small intestine than in the large intestine, causing the difference in time taken for phenotypic changes following mutagen treatment as the mutant stem cell expands stochastically (Figure 36-2A).

An alternative hypothesis might lie in crypt fission: the rate of fission at the time of mutagen administration was higher in the colon than in the small intestine. During crypt fission, when crypts divide longitudinally, selective segregation of the two cell populations could occur, "cleansing" the partially mutated crypts by segregating the mutated and nonmutated cells, and by duplicating the wholly-negative crypts to create monoclonal crypts (Figure 36-3). First proposed as a concept by Park, this phenomenon was illustrated by Bjerknes and Cheng.

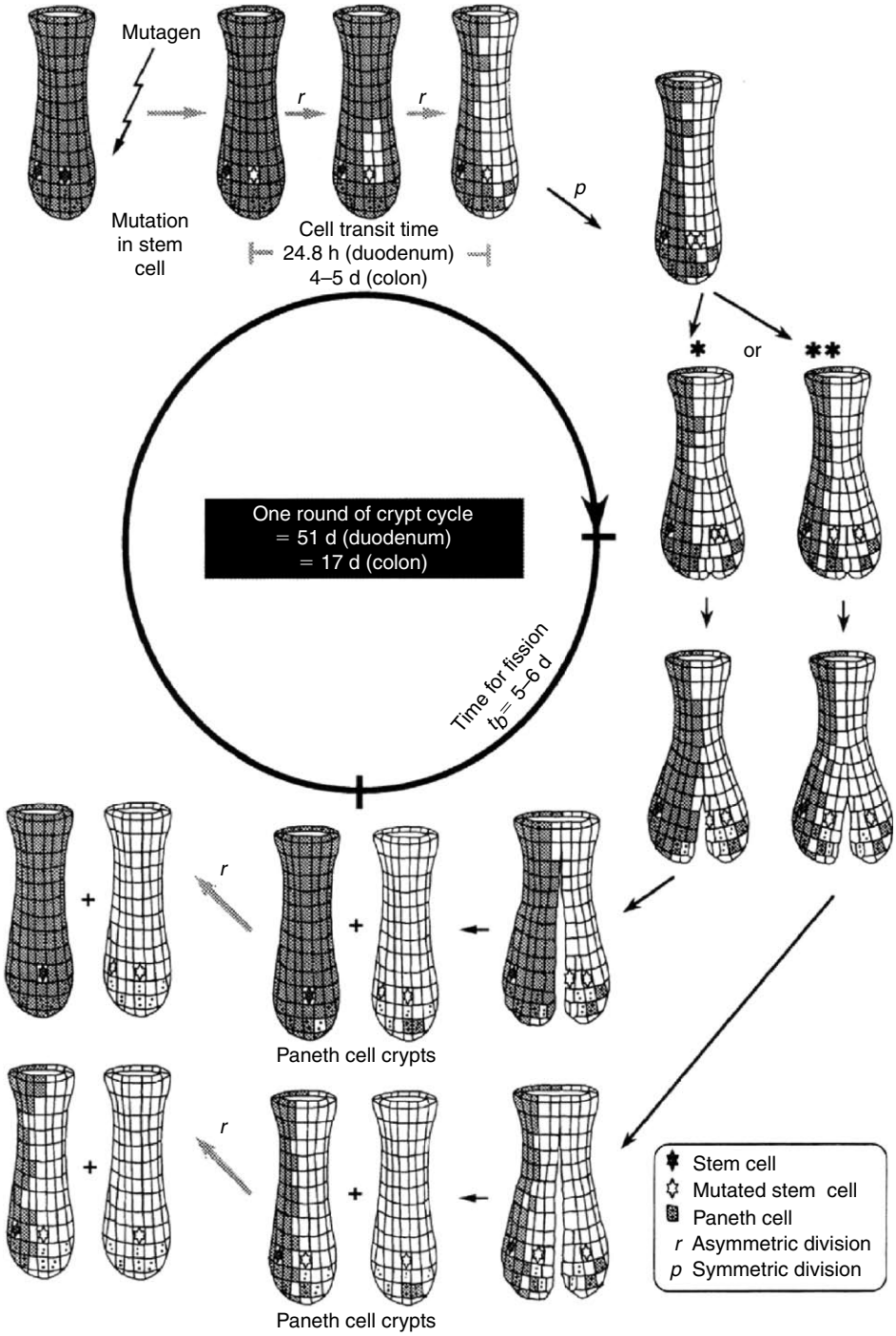


FIGURE 36-3 Crypt cycle. Emergence of transformed crypts after a single stem cell mutation. Crypt division is triggered by symmetric stem cell divisions (p), indicated by light-colored arrows, whereas dark arrows show asymmetric stem cell divisions (r). This is independent of the crypt cycle and enables individual crypts to maintain a constant number of stem cells and to continuously replace differentiated progeny over a crypt cell turnover time, on average. Reproduced with permission from Park.

HUMAN INTESTINAL CRYPTS CONTAIN MULTIPLE EPITHELIAL CELL LINEAGES DERIVED FROM A SINGLE STEM CELL

The investigation of gastrointestinal stem cells in humans has proved difficult. The human colorectal carcinoma cell line HRA19 was derived from a primary adenocarcinoma of the rectum. A colony of single morphology was cloned and grown as a monolayer *in vitro*. When engrafted subcutaneously into *nude* mice, these clones produce tumors histologically-identical to the original tumor and contain columnar, goblet, and neuroendocrine cells (Figure 36-4A–D). These malignant epithelial cells are therefore multipotential, and can produce all differentiated cell types in the human colorectal epithelium. However, these results cannot definitively be applied to normal human gastrointestinal epithelia.

In most of the human population, the colonic goblet cells secrete O-acetylated mucin. However, approximately 9% of the human Caucasian population have a homozygous genetic mutation in the enzyme O-acetyl transferase (*OAT*−/−), and in goblet cells secreting this non-O-acetylated sialic acid; these are then positive when stained with mild periodic acid Schiff (mPAS). In heterozygotes, which comprise approximately 42% of the population (*OAT*−/*OAT*+), O-acetylation proceeds, and crypts thereby stain negative for mPAS. Loss of the remaining active *OAT* gene converts the genotype to *OAT*−/*OAT*−, resulting in occasional, apparently randomly located, positive mPAS-stained crypts with uniform staining of goblet cells from base to luminal surface, an effect that increases with age. This could be because of a somatic mutation or nondisjunction in a single crypt stem cell and subsequent colonization of the crypt by the mutated stem cell. The frequency of these events is racially determined and increases after irradiation. This has been interpreted as indicating an increased rate of stem cell mutation. Interestingly, there is no increase in the rate of apparent stem cell mutation in hereditary nonpolyposis carcinoma patients, nor in the background mucosa of left- and right-sided carcinomas. However, just as in the mouse stem cell mutation models, when patients are followed by the mPAS method with time after irradiation, initially there is partial crypt staining, and then whole crypts appear where the goblet cells are mPAS+. A "clonal stabilization time" (defined as the period required for the emergence of most of such wholly-stained crypts) in humans is approximately one year, a process we referred to previously as monoclonal crypt conversion. These results have implications for the origins of goblet cell lineages in the gut: they indicate that they arise from crypt stem cells; however, they say little about the other cell lineages.

Perhaps the best evidence for the clonality of human intestinal crypts, and the stem cell derivation of all contained epithelial cell lineages, comes from studies of the colon of a rare XO–XY patient who received a prophylactic colectomy for familial adenomatous polyposis (FAP). Nonisotopic *in situ* hybridization (NISH) using Y-chromosome-specific probes showed the patient's normal intestinal crypts to be composed almost entirely of either Y-chromosome positive or Y-chromosome negative cells (Figure 36-4E), with about 20% of crypts being XO. Immunostaining for neuroendocrine specific markers and Y-chromosome NISH, used in combination, showed that crypt neuroendocrine cells shared the genotype of other crypt cells (Figure 36-4F). In the small intestine, the villus epithelium was a mixture of XO and XY cells, in keeping with the belief that the villi derive from stem cells of more than one crypt. Of the 12614 crypts examined, only four crypts were composed of XO and XY cells, which could be explained by nondisjunction with a loss of the Y-chromosome in a crypt stem cell. Importantly, there were no mixed crypts at patch boundaries. These observations agree with previous findings using chimeric mice that intestinal crypt epithelial cells, including neuroendocrine cells, are monoclonal and derive from a single multipotential stem cell. Consequently, the hypothesis that enteroendocrine cells and other differentiated cell types within the colorectal epithelium share a common cell of origin (the Unitarian

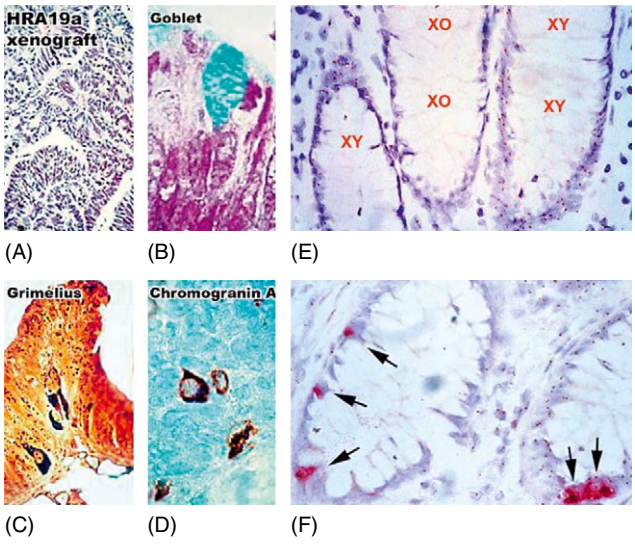


FIGURE 36-4 Monoclonal origin of human clonic crypts. (A) A tumor growing in a nude mouse derived from a single-cell cloned human colorectal carcinoma cell line, stained (B) with Alcian blue to show goblet cells, and with (C) Grimelius and (D) an antibody against chromogranin A to demonstrate endocrine cells. (B) Monoclonal origin of human colonic crypts: normal colonic mucosa in an XO–XY mosaic individual stained by *in situ* hybridization for a Y-chromosome-specific probe showing an XO crypt (central) surrounded by two XY crypts. (C) Endocrine cells, highlighted by their chromogranin A content, also show clonality with the other crypt cell lineages in the same patient. (D) Villi, receiving cells from more than one crypt of different clonal derivation, show a polyclonal pattern in this X0–XY patient. Apart from the occasional Y-chromosome positive inflammatory cell (white arrow), most cells on the right of this villus are XO (black arrows); on the left side, the cells are XY. Panel A–D courtesy of S. Kirkland, panels E and F courtesy of M. Novelli.

hypothesis) appears to apply to both mice and humans. These observations have been confirmed in Sardinian women heterozygous for a defective *G6PD* gene.

Recently it has been shown that colonic stem cells and their progeny contain non-pathogenic mutations in their mitochondrial DNA (mtDNA), specifically mutations affecting the activity of cytochrome *c* oxidase (COX)—a component of complex IV of the respiratory chain, encoded by mtDNA, that are relatively common. The mitochondrial genome is small (16.6 kb) and prone to mutation, due to a lack of protective histones and poor DNA repair mechanisms, in addition to residing in an environment with high oxidation stress. Genetic drift and expansion of a single mutated mtDNA allows mutations to expand stochastically within a cell and over time cells will become either "homoplasmic"—all the mitochondria in the cell are mutated, or "heteroplasmic"—the cell contains a mixture of mutated and wild-type mitochondria. This stochastic expansion is a lengthy process, and COX-deficient cells are rare in young people. For a mutated cellular phenotype to be observed, homoplasmy or a high degree of heteroplasmy must be present, thus stem cells are the only cells that have a sufficient life-span to accumulate these mitochondrial mutations to a level that results in a biochemical deficiency. Two-color enzyme histochemistry can be used to simultaneously detect the mtDNA-encoded cytochrome *c* oxidase and nuclear DNA-encoded succinate dehydrogenase (SDH), a component of complex II of the respiratory chain. Three different types of crypts are observed: wild-type crypts; wholly-mutated crypts; and partially-mutated crypts containing both mutant and wild-type cells. Laser micro-dissection on wholly-mutated crypts, followed by PCR coupled with mtDNA sequencing, confirms the presence of conserved clonal mitochondrial mutations within crypts. This confirms that crypts are clonal populations, as here a crypt has been repopulated from a single mutated stem cell via "niche succession" (the process by which one stem cell takes over the entire stem cell population of a crypt) and "monoclonal conversion" (the process by which the progeny of a single stem cell clone make up the entire crypt). The existence of crypts containing two cell populations, one wild-type and one mutated, demonstrates that human intestinal crypts contain at least two stem cells, and has enabled the dynamic analysis of clonal expansion and identification of the stem cell niche in human colonic tissue. Thus, we now have evidence in human tissue of niche succession and monoclonal conversion "in action," and evidence that multiple stem cells maintain a crypt.

This technology is not only applicable to studying the expansion of clones in the colon, but has also been used as a tool to study the mechanism of field cancerization in the human stomach and duodenum. In particular, the complex structure of the human gastric unit (comprised of multiple glands and a single pit) meant that it has been very difficult to establish the location of the stem cells and the clonal architecture of the human gastric unit. Nomura used X-chromosome-linked inactivation to study fundic and pyloric glands in human female stomachs. Studies using polymorphisms on X-linked genes, such as the androgen receptor (HUMARA) to distinguish between the two X-chromosomes revealed that, although pyloric glands appear homotypic and thus monoclonal, about half of the fundic glands studied were heterotypic for at the HUMARA locus, and were consequently polyclonal. This finding suggests that a more complex situation occurs in humans than the studies of gastric gland clonality in chimeric mice indicate. However, we have seen that some glands in the mouse remain polyclonal throughout life. Using the COX model, the authors were able to demonstrate that human gastric units are clonal and that they are able to divide by gland fission, forming entirely clonal patches of mutated gastric units: this strong evidence to support fission as a mechanism of field cancerization.

It has been proposed that insight into stem cell organization can be gained from the study of the methylation pattern of nonexpressed genes in the colon. In the normal human colon, methylation patterns are somatically-inherited endogenous sequences that randomly change and increase in occurrence with aging. Investigation of methylation patterns is a possible alternative to histological markers to investigate crypt histories and allow fate mapping. Examination of methylation tags of three neutral loci in cells from normal human colon showed variation in sequences between crypts and mosaic methylation patterns within single crypts. Multiple unique sites were present in morphologically identical crypts; for example, one patient had no identical methylation sequences of one gene within any of the crypts studied, even though all sequences were related. This indicates that some normal human colonic crypts are quasi-clonal, with multiple stem cells per crypt. Differences in methylation tags can highlight relationships among cells in a crypt where less closely-related cells show greater sequence alterations, and where closely-related cells have similar methylation patterns. Sequence differences suggest that crypts are maintained by stem cells, which are randomly lost and replaced in a stochastic manner, eventually leading to a "bottleneck" effect, in which all cells within a crypt are closely-related to a single stem cell descendant. This reduction to the most recent common crypt progenitor is predicted to occur several times during life, superficially resembling the clonal succession of tumor progression.

BONE MARROW STEM CELLS CONTRIBUTE TO GUT REPOPULATION AFTER DAMAGE

The haematopoietic bone marrow stem cell is of mesodermal origin, and its functionality and cell surface markers

have been well-characterized. When transplanted into lethally irradiated animals and humans, as in clinical bone marrow transplantation, it has long been considered that the haematopoietic stem cell colonizes host tissues to form only new erythroid, granulocyte–macrophage, megakaryocyte, and lymphoid lineages. Although earlier studies suggest that vascular endothelium could derive from transplanted donor marrow, other studies have not only confirmed these earlier proposals concerning endothelial cells, but have also indicated that adult bone marrow stem cells possess a considerable degree of plasticity and can differentiate into different cell types, including hepatocytes, biliary epithelial cells, skeletal muscle fibres, cardiomyocytes, central nervous system cells, and renal tubular epithelial cells. These pathways can be bidirectional, as muscle and neuronal stem cells can also apparently form bone marrow. Furthermore, it appears that selection pressure induced by target organ damage can intensify the efficacy of this process as bone marrow stem cells differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells in mice, with ischemic cell death following myocardial infarction and coronary artery occlusion. Bone marrow stem cells have also been shown to differentiate into pancreatic β-cells, and possibly more persuasively, fully-differentiated cells can transdifferentiate into other adult cell types without undergoing cell division; for example, exocrine pancreatic cells can differentiate into hepatocytes *in vitro*. Furthermore, isolated potential hepatic stem cells from fetal mouse livers, which differentiate into hepatocytes and cholangiocytes when transplanted into recipient animals, can form pancreatic ductal acinar cells and intestinal epithelial cells when transplanted directly into the pancreas or duodenal wall. Thus, the conventional view that bone marrow stem cells generate cell types of a single lineage (i.e., all formed elements in the peripheral blood) has been rectified in favor of findings that adult bone marrow stem cells are highly plastic, and can differentiate into many cell types within various organs.

These observations have raised the possibility of regeneration of a failing organ by transplanting an individual's own bone marrow stem cells to colonize and repopulate the diseased tissue, thus avoiding the allograft reaction. In apparent proof-of-principle, fumarylacetoacetate hydrolase (FAH)-deficient mice, which resemble Type 1 tyrosinemia in humans, are rescued from liver failure by transplantation of purified hematopoietic stem cells that become morphologically normal hepatocytes, express the FAH enzyme, and therefore are functionally normal.

There are now several reports that bone marrow cells can repopulate both epithelial and mesenchymal lineages in the gut. Brittan analyzed colons and small intestines of female mice that received a bone marrow transplant from male mice donors, and gastrointestinal biopsies from female patients with graft-versus-host disease following bone marrow transplant from male donors. Bone marrow cells frequently engraft into the mouse small intestine and colon, and differentiate to form ISEMFs within the lamina propria. *In situ* hybridization confirmed the presence of Y-chromosomes in these cells; their positive immunostaining for αSMA and negativity for desmin, the mouse macrophage marker F4/80, and the hematopoietic precursor marker CD34 determined their phenotype as pericryptal myofibroblasts in the lamina propria derived from transplanted bone marrow. This engraftment and transdifferentiation occurred as early as one week after bone marrow transplantation; almost 60% of ISEMFs were bone marrow-derived six weeks after the transplantation, indicating that transplanted bone marrow cells are capable of a sustained turnover of the ISEMF cells in the lamina propria (Figure 36-5). Y-chromosome-positive ISEMFs were also seen in the human intestinal biopsy material. Lethally-irradiated female mice given a male bone marrow transplant and a subsequent foreign-body peritoneal implant formed granulation tissue capsules containing myofibroblast cells derived from the hematopoietic stem cells of the transplanted bone marrow. This suggests that myofibroblasts may generally derive from bone marrow cells.

There are a growing number of reports that bone marrow cells can repopulate gastrointestinal epithelial cells in animals and man. Krause found bone marrow-derived epithelial cells in the lung, gastrointestinal tract, and skin 11 months after transplantation of a single hematopoietic bone marrow stem cell in the mouse. In the gastrointestinal

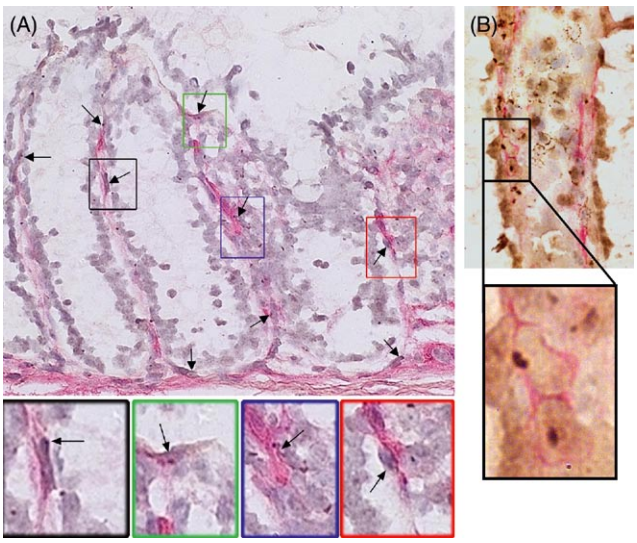


FIGURE 36-5 Bone marrow-derived ISEMFs in the lamina propria of the mouse colon. Female mouse colon following a male whole bone marrow transplant. Bone marrow-derived ISEMFs are present as Y-chromosome-positive cells, immunoreactive for α-SMA (A) two weeks after transplant (arrows and high-power inserts) and as columns reaching from crypt base to tip (B) six weeks after transplant (arrows and high-power inserts). The Y-chromosome is seen as a brown–black punctate density, and red cytoplasmic staining indicates immunoreactivity for α-SMA. Courtesy of M. Brittan.

tract, engrafted cells were present as columnar epithelial cells in the esophageal lining, a small intestinal villus, colonic crypts, and gastric foveola. No apparent engraftment into the pericryptal myofibroblast sheath was reported, and as only a single hematopoietic stem cell was transplanted, it is possible that the ISEMFs derive from mesenchymal stem cells within transplanted whole bone marrow. It is, however, generally believed that stromal cell populations do not survive following bone marrow transplantation—although if an empty niche exists, as after irradiation in the gut, engraftment might occur.

In biopsies from female patients who had undergone sex-mismatched hematopoietic bone marrow transplantation, *in situ* hybridization for a Y-chromosome-specific probe with immunohistochemical staining for cytokeratins demonstrated mucosal cells of donor origin in the gastric cardia. Moreover, Okamoto reported four long-term bone marrow transplant survivors with multiple engraftment of esophageal, gastric, small intestinal, and colonic epithelial cells by donor bone marrow cells up to eight years after transplantation, emphasizing the long-term nature of this transdifferentiation.

It would be impossible to finish even such a short section on this topic without mentioning the mechanisms and significance of such phenomena. At first considered to be caused by transdifferentiation, or lack of lineage fidelity—commonly seen in invertebrates during gastrulation, or during organogenesis—it is becoming clear that such changes are neither simple nor readily reproducible. Some laboratories have been unable to reproduce earlier findings, and there are claims that adult tissues are contaminated with bone marrow precursors. Finally, the fusion of a transplanted bone marrow cell with an indigenous adult cell has been proposed as the mechanism by which bone marrow stem cells acquire the phenotype of target cell lineages. Initially, cell fusion was seen as a rare event, occurring only *in vitro*, and under circumstances of extreme selection. In the FAH model described previously, cell fusion has been shown to be common, and it cannot be ruled out as the main mechanism by which bone marrow stem cells transform to functional hepatocytes. However, the genetic mechanisms whereby gene expression is switched off in the recipient cells—followed by clonal expansion to repopulate large areas of the liver, for example—are as yet unclear. We should also recall that several tissues in the mouse are polyploid, such as the liver and the acinar cells of the exocrine pancreas. Other studies in which mixed-sex bone marrow transplants have been used to show plasticity have not reported evidence of cell fusion in animals or man: for example, bone marrow-engrafted cells in the human stomach, intestine, buccal mucosa, and pancreatic islet cells showed a normal complement of X- and Y-chromosomes. Whatever the mechanism, it is clear that the most important criterion for altered lineage commitment, that of function, has only been fulfilled in a few models, such as the FAH model and possibly postinfarction cardiomyocyte engraftment. The future in this field will prove interesting.

In inflammatory bowel diseases such as Crohn's disease, intestinal myofibroblasts are activated to proliferate and synthesize an extracellular matrix, and excessive collagen deposition causes fibrosis and postinflammatory scarring in the lamina propria and muscularis layers of the gut wall. Intestinal inflammation is believed to be mediated by luminal bacteria and bacterial wall polymers, and tumor necrosis factor (TNF) plays a key role in the pathogenesis of intestinal inflammatory disease, since mice with targeted deletion of the AU-rich elements in TNF develop chronic ileitis resembling a Crohn's disease-like phenotype (Figure 36-6A–D). Moreover, a single dose of anti-TNF antibody to Crohn's disease patients can dramatically alleviate inflammation. Other cytokines, including interleukin-10 and TGFβ are implicated in the development of fibrosis in inflammatory bowel disease.

It has previously been concluded that fibrotic reactions, which occur in inflammatory bowel disease and other diseases, are caused by the local proliferation of myofibroblasts and fibroblasts. Our data showing that transplanted bone marrow contributes to the intestinal subepithelial myofibroblast population implicates that extraintestinal cells may have a role in fibrosis. In this respect, we have shown that cells with a fibroblast–fibrocyte phenotype can derive from transplanted bone marrow, and contribute to fibrotic reactions in and around the intestinal wall (Figure 36-6E). The concept that cells of the lamina propria, other than the lymphoid and myeloid lineages, exist in equilibrium with bone marrow precursors is an interesting one and could provide an opportunity for therapeutic delivery of cytokines to the intestine to prevent the development of fibrosis, or even to treat it. Further data show that many of the myofibroblasts and fibroblasts recruited to, or expanding in, the lamina propria and submucosa of mice with induced TNBS colitis are bone marrow-derived (Figure 36-6F).

The IL-10 knockout mouse is a well-established model of colitis. Bamba transplanted female IL-10−/− mice with either male IL-10 bone marrow or wild-type bone marrow. In the former group, a florid colitis developed with up to 45% of myofibroblasts being bone marrow-derived. However, IL-10−/− mice displayed an ameliorated course of disease after wild-type bone marrow transplant with up to 30% of myofibroblasts being of donor origin. Subsequently, work in patients with Crohn's disease has shown an improvement in disease course with CD34 enriched and hematopoietic stem cells, as well as mobilized peripheral blood stem cells. In these cases the therapeutic effect is postulated to be due to the immunosuppressive effects of the conditioning regimen required for stem cell transplant. The autologous stem cell transplant serves to shorten the neutropenic interval, rather than reconstitute the myofibroblast component of the colonic niche—a mechanism that has not as yet been proven.

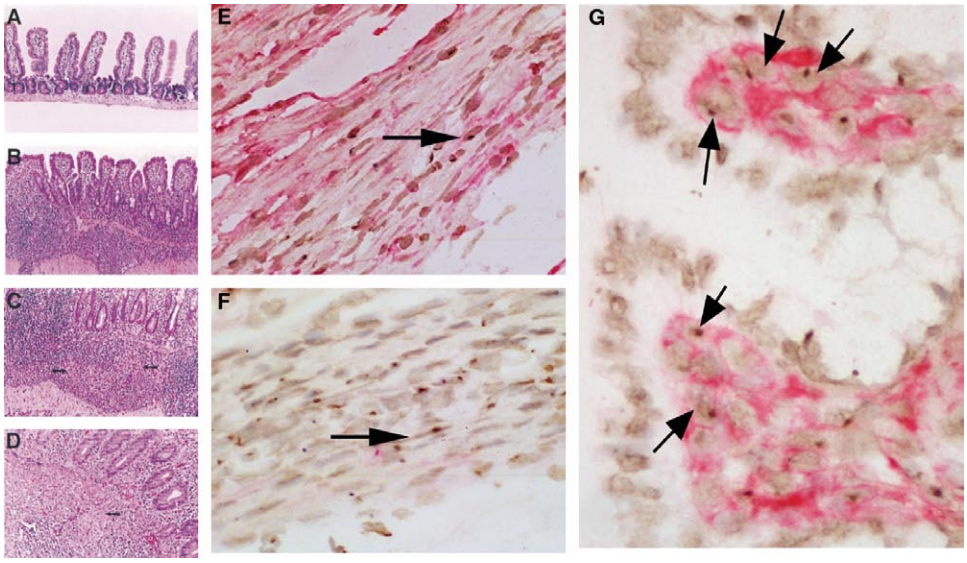


FIGURE 36–6. Murine models of fibrosis. Inflammatory bowel disease in $TNF^{\Delta ARE}$ and SAMP/Yit mouse ilea resembling human Crohn's disease. (A) Normal ileal morphology in an 8-week control mouse. (B) $TNF^{\Delta ARE}$ homozygous knockout at 7 weeks with blunt, distorted villi and increased inflammatory infiltrate. (C) $TNF^{\Delta ARE}$ heterozygote at 16 weeks with villus blunting and chronic inflammation. An ill-defined noncaseating granuloma is in the submucosa (arrows). (D) Ileocecal region from a human patient with Crohn's disease demonstrating similar location and composition of granuloma compared with mouse model (arrow). (E) A fibrotic reaction in the serosal tissues of a paracetmol-treated male–female radiation chimeric mouse, showing numerous spindle-shaped vimentin-positive cells with Y-chromosomes revealing their bone marrow origin, which are also (F) smooth muscle actin negative, confirming their fibroblast lineage. (G) Massive expansion of myofibroblasts of bone marrow origin in a male–female chimeric mouse with colitis induced by TNBS. Panel D reproduced with permission from Kontoyiannis, panels E–F courtesy of N. Direkze, and panel G courtesy of M. Brittan.

GASTROINTESTINAL STEM CELLS OCCUPY A NICHE MAINTAINED BY INTESTINAL SUBEPITHELIAL MYOFIBROBLASTS IN THE LAMINA PROPRIA

Stem cells within many tissues are thought to reside within a niche formed by a group of surrounding cells and their extracellular matrices, which provide an optimal microenvironment for the stem cells to function. The identification of a niche within any tissue involves knowledge of the location of the stem cells; as we have seen, this has proved problematical in the gastrointestinal tract. According to Spradling, to prove that a niche is present, the stem cells must be removed and subsequently replaced while the niche persists, providing support to the remaining exogenous cells. Although this has been accomplished in *Drosophila*, such manipulations have not yet been possible in mammals. In this context, the survival of a single epithelial cell following cytotoxic damage to intestinal crypts in the microcolony assay is interesting, as many of the intestinal subepithelial myofibroblasts are also lost after irradiation, although sufficient numbers may remain or be replaced by local proliferation or migration from the bone marrow to provide a supportive niche for the surviving stem cell or cells. The ISEMFs surround the base of the crypt and the neck–isthmus of the gastric gland, a commonly proposed location for the intestinal and gastric stem cell niches, respectively. It is proposed that ISEMFs influence epithelial cell proliferation and regeneration through epithelial–mesenchymal cross talk, and that they ultimately determine epithelial cell fate.

There has been a long quest for markers of stem cells in the intestine. The neural RNA-binding protein marker Musashi-1 (Msi-1) is a mammalian homolog of a *Drosophila* protein evidently required for asymmetrical division of sensory neural precursors. In the mouse, Msi-1 is expressed in neural stem cells and has been proposed as the first intestinal stem cell marker, because of its expression in developing intestinal crypts and specifically within the stem cell region of adult small intestinal crypts. This is further substantiated by its expanded expression throughout the entire clonogenic region in the small intestine after irradiation.

The regulatory mechanisms of stem cell division within the niche to produce, on average, one stem cell and one cell committed to differentiation is as yet unknown, although there is no shortage of potential models. In the stem cell zone hypothesis, the bottom few cell positions of the small intestinal crypt are occupied by a mixture of cell types: Paneth, goblet, and endocrine. The migration vector is towards the bottom of the crypt. Above cell position 5, cells migrate upwards, although only the cells that divide in the stem cell zone beneath are stem cells. Other models envisage the stem cells occupying a ring immediately above the Paneth cells, although there is little experimental

basis for such an assertion since "undifferentiated" cells of similar appearance are seen among the Paneth cells in thin sections. Moreover, there is no difference in the expression of Msi-1 and Hes1—a transcriptional factor regulated by the Notch signaling pathway also required for neural stem cell renewal and neuronal lineage commitment—in undifferentiated cells located in either the stem cell zone or immediately above the Paneth cells. This suggests that both populations may have the same potential as putative stem cells.

The number of stem cells in a crypt or a gland is presently unknown. Initially, all proliferating cells were believed to be stem cells. Although clonal regeneration experiments using the microcolony assay indicated that intestinal crypts contained a multiplicity of stem cells, it was clear that this was less than the proliferative cellularity. Proposed stem cell numbers have varied from a single stem cell to 16 or more. Others have proposed that the number of stem cells per crypt varies throughout the crypt cycle, with the attainment of a "threshold number" of stem cells per crypt being the signal for fission to occur. There is little experimental evidence to support these proposals. Although all cells in a crypt are initially derived from a single cell, as shown by the X-inactivation experiments discussed previously, mutagenesis studies such as those shown in Figure 36-1A argue strongly for more than one stem cell per crypt, with stochastic clonal expansion of a mutant clone. A three stem cell colonic crypt has been suggested by Williams on this basis.

In organisms such as *Drosophila* and *Caenorhabditis elegans*, stem cell divisions are known to be asymmetric. We have no such firm concept in the mammalian gut, although there is some evidence to support the proposal. By labeling DNA template strands in intestinal stem cells with tritiated thymidine during development or tissue regeneration, and by labeling newly-synthesized daughter strands with bromodeoxyuridine, segregation of the two markers can be studied. The template DNA strand labeled with tritiated thymidine is retained, but the newly-synthesized strands labeled with bromodeoxyuridine become lost after the second division of the stem cell. This indicates not only that asymmetric stem cell division occurs, but also that by discarding the newly-synthesized DNA which is prone to mutation into the daughter cell destined to differentiate, a mechanism of stem cell genome protection is afforded.

When a stem cell divides, the possible outcomes are that two stem cells (P) are produced, that two daughter cells destined to differentiate cells (Q) are produced, or that there could be an asymmetric division resulting in one P and one Q cell. These are sometimes called p, q, and r divisions, or p and q divisions (Figure 36-7). If $p = 1$ and $q = 0$, then regardless of the number of stem cells per crypt, the cells are immortal and there will be no drift in the niche with time. Such a situation is called "deterministic." However, if $p < 1$ and $Q > 0$ (i.e., a stochastic model), there will be eventual extinction of some stem cell lines, and a drift towards a common stem cell from which all other cells derive. We previously described the variation in methylation patterns or "tags" that occur in human colonic crypts, and explained that crypts apparently show several unique tags. The variance in these unique tags was compared with those expected using a variety of models, including no drift with aging (the deterministic model), drift with immortal stem cells with divergence (the numbers of unique tags are proportional to the stem cell number), drift with one stem cell per crypt, and a stem cell niche with more divergence (with loss of stem cells occurring proportional to the time since divergence). Multiple unique tags were found in some crypts, and the number of unique tags increased with the number of markers counted,

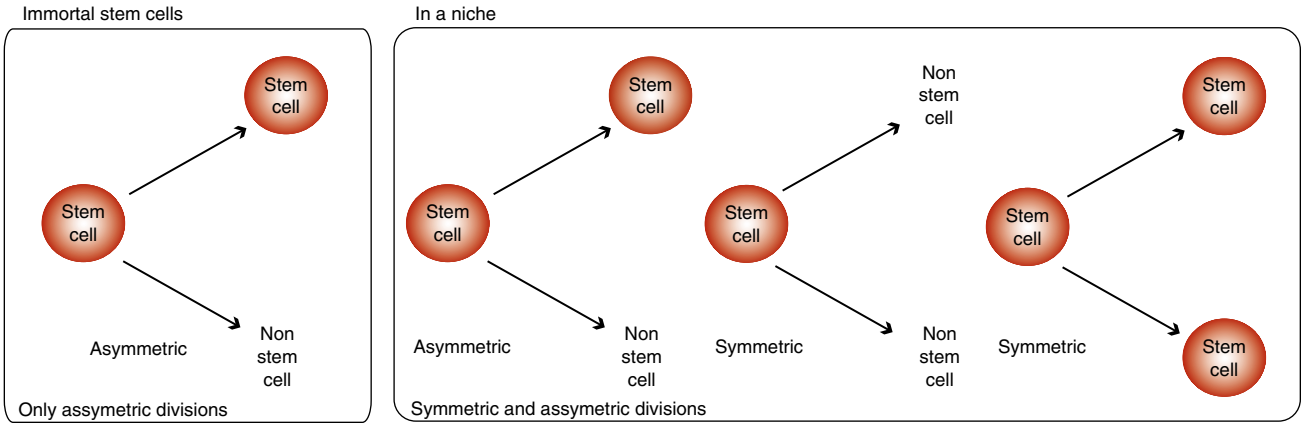


FIGURE 36-7 How stem cells divide in a niche. Classically, stem cells always replace themselves by asymmetrical division; therefore, their lineages never become extinct. However, although the stem cell number remains constant in a niche, both asymmetrical and symmetrical divisions occur. Symmetrical division can lead to one stem cell lineage becoming extinct as both daughters leave the niche, but to maintain a constant stem cell number in the niche, this loss is balanced by stem cell expansion, where both daughters remain in the niche as stem cells. Such stem cell loss with ensuing replacement can lead to the extinction of all lineages bar one, or *clonal succession.* Reproduced with permission from Kim.

which favors random tag drift and multiple stem cells per crypt. The variances were consistent with drift in immortal stem cells, where N (the number of stem cells) = 2, but favored a model where $0.75 < P < 0.95$, and $N < 512$. Thus, the data supported a stochastic model with multiple stem cells per crypt. However, as in many such attempts, there are several major assumptions necessary, such as a constant stem cell number. It is clear that variation in both P and N occur in this model. However, this analysis is consistent with the results of Campbell, who deduced that the time taken for monoclonal conversion, or the "clonal stabilization time," of OAT+/− individuals to convert to OAT−/− cells following irradiation was found to be about one year. Assuming 64 stem cells per niche, and $P = 0.95$, the mean time for conversion should be some 220 days. The same assumptions suggest a bottleneck, where all stem cells are related to the most recent common ancestral cell, occurring every 8.2 years.

MANY SIGNALING PATHWAYS REGULATE GASTROINTESTINAL DEVELOPMENT, PROLIFERATION, AND DIFFERENTIATION

Although the molecular mechanisms by which pluripotent stem cells of the gastrointestinal tract produce differentiated cell types are not entirely understood, considerable progress has been made in the last few years. An increasing number of genes and growth factors have been identified that regulate development, proliferation, and differentiation, as well as the development of tumors. The Wnt/β-catenin pathway appears to be a crucial mechanism in regulating proliferation, and constitutionally active Wnt signaling is sufficient to initiate tumor growth. Other pathways, including the Notch pathway and bone morphogenic protein (BMP) pathways and Hedgehog signaling, have also been shown to influence intestinal crypt cell proliferation and differentiation. The intestinal mesenchymal and epithelial cells themselves express these molecules. Details of these pathways are described in the following sections.

WNT/β-CATENIN SIGNALING PATHWAY MAINTAINS THE PROLIFERATIVE COMPARTMENT OF INTESTINAL CRYPTS

The Wnt family of signaling proteins is critical during embryonic development and organogenesis in many species. There are 19 known murine *Wnt* genes, which bind to receptors of the frizzled (*Fz*) family, 10 of which have been identified in mice, and to the low-density lipoprotein receptor-related proteins (*LRP*). Canonical Wnt signaling is mediated by the multifunctional protein β-catenin. β-catenin normally interacts with the so-called "destruction complex," a complex of glycogen synthase kinase 3-β (GSK3-β), axin, and the adenomatous polyposis coli (APC) tumor-suppressor protein. Subsequent serine phosphorylation of cytosolic β-catenin by GSK3-β leads to its ubiquitination, and to its proteasomal degradation, thereby maintaining low levels of cytosolic and nuclear β-catenin. Wnt ligand binding to its cell surface receptors activates the cytoplasmic phosphoprotein "disheveled," which in turn initiates a signaling cascade resulting in the inhibition of the destruction complex and increased cytosolic levels of β-catenin. The free β-catenin then translocates to the cell nucleus, where it forms a transcriptional activator by combining with members of the T-cell factor/lymphocyte enhancer factor (Tcf/LEF) DNA-binding protein family. This activates transcription of specific genes, including *c-myc*, *cyclin D1*, *CD44*, *c-Jun*, *Fra-1*, and the putative stem cell marker *Lgr5*, and results in cell proliferation (see Figure 36-8).

β-catenin/Tcf signaling is required for maintenance of the proliferative compartment of the intestinal crypt. Mice with targeted disruption of *Tcf-4* have no proliferating cells within their small intestinal crypts, and lack a functional stem cell compartment. Similarly, conditional ectopic expression of Dickkopf-1 in the intestine of adult mice, a potent Wnt inhibitor, has similar effects. In colorectal cancer cells, disruption of β-catenin/TCF signaling causes rapid cell cycle arrest. Furthermore, induced deletion of the Wnt target gene *c-myc* in adult mice causes rapid loss of intestinal crypts. Gregorieff, using *in situ* hybridization, showed the distribution of wnts, and their receptors and antagonists, along the major axis of murine intestinal crypts. A number of Wnts, and their receptors and antagonists, show differential expression along the crypt axis: in the small intestine for example, Wnt-3, Wnt-6, and Wnt-9b are expressed predominantly at the crypt base. As well as maintaining a proliferative compartment, Wnt signals also appear to be involved in inducing cell differentiation: conditional deletion of the *Frizzled-5* receptor gene in mice resulted in Paneth cells failing to home to the crypt base.

As well as being required to maintain homeostasis in the healthy intestine, the Wnt/β-catenin pathway plays a key role in malignant transformation. Mutations of the *APC* tumor suppression gene, which typically result in a truncated protein product, are present in up to 80% of human sporadic colorectal tumors. This mutation inhibits cytosolic β-catenin degradation by the GSK3β/axin/APC complex. This results in increased nuclear β-*catenin*, and a subsequent increase in β-catenin-induced Tcf/LEF transcription. Thus, one of the main tumor suppressing functions of *APC* is the destabilization of β-catenin. Indeed, accumulation of nuclear β-catenin is one of the earliest events, or perhaps even *the* initiating event, in human and mouse intestinal tumorigenesis. This majority of the tumorigenic Wnt signaling may be mediated by the Wnt target

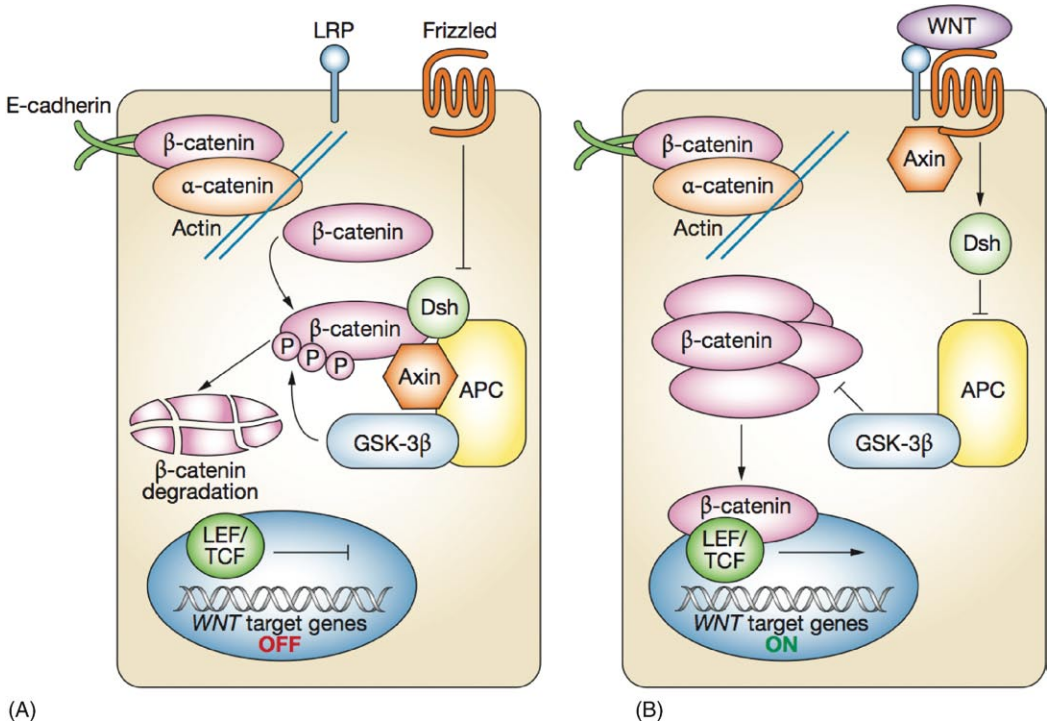


FIGURE 36-8. The Wnt signalling pathway. (A) In the absence of WNT bonding to frizzled, dishevelled remains unactivated and B-catenin is tagetted for degradation via the adenomatous polyposis coli/axin/glycogen-synthase-kinase 3Bpathway. (B) When Wnt binds to frizzled (right), dishevelled is activated and uncouples B-catenin from the adenomatous polyposis coli complex: B-catenin can then associate with transcription factors and mediate transcription of the target gene. APC, adenomatous polyposis coli; Dsh, dishevelled; GSK-3B, glycogen synthase kinase 3B; LEF, lymphoid enhancer-binding factor; LRP, LDL-receptor related protein; P, phosphorylation; TCF, T cell factor.

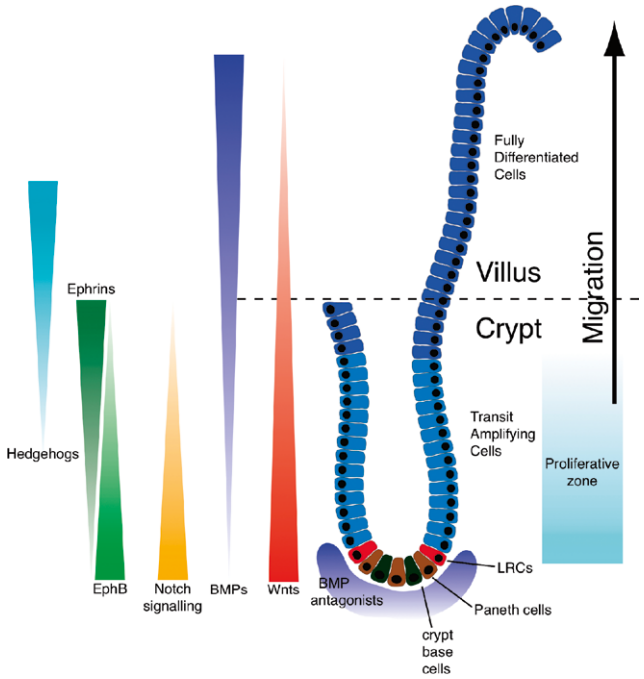


FIGURE 36-9 Schematic of signaling pathways in intestinal crypts. Proliferation tends to be restricted to the crypt base, where Wnt signals are most strongly expressed. BMP signaling increases away from the crypt base, whereas the BMP-antagonists, which include Germlins and Noggin, are principally expressed in the ISEMFs at the crypt base. The hedgehog molecules, antagonists of Wnt signaling, are chiefly expressed at the crypt tops.

c-myc, as simultaneous disruption of both *APC* and *c-myc*, restricts the tumor growth induced by *APC* disruption. Interestingly, the level of Wnt signaling required for tumor cell growth appears to be finely-tuned: tumor cells appear to select for "just-right" levels of β-catenin/TCF signaling. Tumorigenic APC mutations tend to result in a truncated protein product, which tends to retain one, or less frequently, two or three, of the 20 amino acid β-catenin-binding domains. The truncated APC protein can still regulate β-catenin activity, suggesting that there is selection for an optimal level of Wnt signaling during tumor growth. Furthermore, Segditsas and colleagues recently reported "third-hits" in APC in human colorectal cancers, suggesting that the Wnt pathway may be subjected to repeated targeting during cancer development. These third-hits may be selected for on the basis that they provide a new optimal level of Wnt-signaling for the cancer cells, which may be different to the Wnt-signal that provided an optimal proliferation signal in the early lesion.

WNT TARGETS EPHB DETERMINE CELL POSITIONING ALONG THE CRYPT AXIS

The Wnt targets, the receptor tyrosine kinases *EphB2* and *EphB3* are required for the correct positioning of epithelial cells along the crypt axis. In EphB3−/− knockout mice, for

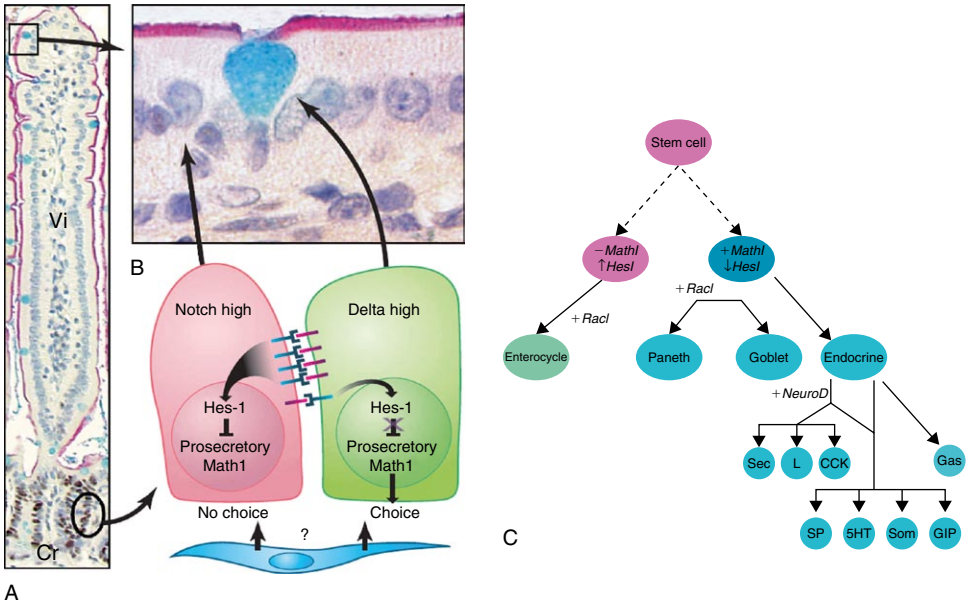


FIGURE 36-10 Math1 signaling pathway. (A) Low-power section of adult murine small intestine. Precursor cells are stained for cyclin proliferating cell nuclear antigen, enterocytes express intestinal alkaline phosphatase, and goblet cells secrete mucins. Inset shows high-power image of small intestinal enterocytes and goblet cells. (B) Math1, a component of the Notch signaling pathway, influences intestinal epithelial cell fate decisions. In crypt progenitor stem cells that express high levels of Notch, the Hes1 transcription factor is switched on, and the expression of Math1 and of other "prosecretory" genes is blocked. The result is that the precursor cells become enterocytes. In cells expressing low amounts of Notch, levels of Delta are high, production of Hes1 is blocked, and Math1 expression is induced. Production of the Math1 helix–loop–helix transcription factor allows precursor cells to make a choice: whether to become goblet cells, Paneth cells, or enteroendocrine cells (C) Math1 is essential for secretory cells. Whether Math1-expressing cells descend directly from stem cells or an intermediate progenitor remains unknown. (Vi: villus, Cr: crypt, Sec: secretin, L: glucagon–peptide YY, CCK: cholecystokinin, SP: substance P, 5HT: serotonin, Som: somatostatin, GIP: gastric inhibitory peptide, and Gas: gastrin). Panels A and B reproduced with permission from van Den Brink and panel C reproduced with permission from Yang.

example, Paneth cells do not migrate downwards to their normal position at the bottom of the crypt, but instead scatter along crypt and villus. EphB2 and EphB3 receptor expression is strongest at the crypt base, whereas their ligand ephrin-B1 is expressed most strongly at the crypt top (Figure 36-10). The expression of EphB receptors is increased in response to elevated Wnt-signaling. Interestingly, EphB receptor expression is lost in human colorectal cancers, and inducible deletion of *EphB* in *ApcMin/+* mice accelerates tumorigenesis and leads to the development of invasive carcinomas. This suggests that EphB mediate a pathway crucial in permitting tumor cells to become invasive.

NOTCH SIGNALING IS ALSO TO MAINTAIN THE PROLIFERATIVE COMPARTMENT OF INTESTINAL CRYPTS, AND DETERMINES CELL FATE

Notch receptors, the ligand delta, and the associated Hes transcription factors are chiefly expressed at the base of intestinal crypts. Binding of the ligand to the Notch receptor causes the cleavage of part of the Notch receptor. The resulting free intra-cellular domain of the receptor translocates to the nucleus, where it binds to the transcription factor CSL/RBP-J and activates target gene transcription. Constitutive Notch signaling is required to maintain the proliferative compartment of intestinal crypts: intraperitoneal injection of mice with a γ-secretase inhibitor, which prevents the cleavage of the activated Notch receptor, rapidly transformed the proliferative cells in the crypt to post-mitotic Goblet cells. Similar results were observed following conditional removal of the CSL/RBP-J transcription factor in adult mice. The requirement for Notch signaling in order to maintain cell proliferation in intestinal crypts is maintained during intestinal tumorigenesis: γ-secretase inhibition in *ApcMin/+* mice caused widespread Goblet cell conversion within adenomas.

In mice, deletion of the *Math1* gene, a basic helix–loop–helix transcription factor and downstream component of the *Notch* signaling pathway, depletes goblet, Paneth, and enteroendocrine cell lineages in the small intestine. Math1− progenitors merely become enterocytes. This indicates that *Math1*, and indeed Notch signaling, is essential for determining stem cell fate (Figure 36-10). High levels of Notch switch on the Hes1 transcriptional repressor, which in turn blocks expression of Math1 so that cells remain progenitors and ultimately become enterocytes. Conversely, low Notch expression increases levels of its ligand Delta, which induces Math1 expression by blocking

Hes1, causing cells to become goblet cells, Paneth cells, or enteroendocrine cells. Correspondingly, Hes1-null mice have elevated Math1 expression, with increased enteroendocrine and goblet cells and fewer enterocytes. Conditional activation of a constitutionally active form of Notch causes a rapid and temporary increase in the number of Goblet cells in the crypt.

BONE MORPHOGENIC PATHWAY SIGNALING REGULATES PROLIFERATION ALONG THE INTESTINAL CRYPT AXIS

The bone morphogenic pathway (BMP) is part of the transforming growth factor β superfamily, and involves many ligands and receptors. BMPs bind to their receptors on the cell surface, causing a signal to be transduced to the nucleus via Smad proteins (crucially Smad4), where they regulate gene expression. Expression of BMPs is highest at the top (luminal end) of intestinal crypts, primarily in the mesenchyme, whereas BMP antagonists, including Gremlin 1 and 2 and Noggin, are expressed strongly at the crypt base (Figure 36-9).

BMPs appear to antagonize Wnt signaling, via the PTEN tumor-suppressor protein, and so permit differentiation and restrict proliferation. Conditional inactivation of the BMPR1A receptor in the mouse intestine resulted in increased cell proliferation. Inhibition of BMP signaling by conditional ectopic expression of the BMP-antagonist Noggin also increased proliferation, and led to *de novo* crypt formation and eventual polyposis. Gremlin 1, a BMP antagonist, was shown to partially inhibit cell differentiation *in vitro* and promote proliferation. Conversely, stimulation of colon cell lines by BMP2 inhibited growth. BMP signaling is also implicated in intestinal tumorigenesis, further illustrating the likely importance of the pathway in the regulation of proliferation in intestinal crypts. The rare inherited syndrome juvenile polyposis is associated with both germline mutations in the *BMPR1A* receptor, and with mutations in the *Smad4* gene. A recent genome-wide association study found that common genetic variants in BMP4 and upstream of BMP2, and in SMAD7, are associated with increased risk of colon cancer.

HEDGEHOG SIGNALING PATTERNS THE CRYPT AXIS

Hedgehog (Hh) signaling appears to work in the opposite direction to the other pathways discussed above. Expression of the two hedgehog ligands Indian and Sonic hedgehog tends to be restricted to the epithelium, and is predominantly in the mature colonocytes at the crypt top, whereas Hh receptors are expressed by the adjacent mesenchyme. Hedgehog signaling is an antagonist of Wnt signaling: transfection of Indian hh (Ihh) into colon cancer cells down-regulates TCF4 expression, whereas conversely, inhibition of TCF4 into colon cancer cells with constitutionally active Wnt-signaling restores Ihh expression. Thus, the differential expression of Hh along the crypt axis appears to restrict Wnt signaling to the crypt base. This localization of Hh is crucial for the correct positioning and maturation of colonocytes: reduction in Hedgehog signaling by expression of a pan-Hh inhibitor in transgenic mice causes epithelial remodeling, and mislocation of the subepithelial myofibroblasts. Differentiation was also disrupted when rats were treated with the pan-Hh inhibitor.

HOX GENES DEFINE REGIONAL GUT SPECIFICATION

Mammalian homeobox genes *Cdx-1* and *Cdx-2* display specific regional expression in developing and mature colon and small intestine. During embryogenesis, *Cdx-1* localizes to the proliferating cells of the crypts, and maintains this expression during adulthood. The Tcf-4 knockout mouse does not express *Cdx-1* in the small intestinal epithelium, thus the Wnt/β-catenin complex appears to induce *Cdx-1* transcription in association with Tcf-4 during the development of intestinal crypts. Mice heterozygous for a *Cdx-2* mutation develop colonic polyps composed of squamous, body, and antral gastric mucosa with small intestinal tissue. Proliferation of *Cdx-2*−colonic cells with low *Cdx-2* levels can produce clones of cells phenotypically similar to epithelial cells of the stomach or small intestine. This could indicate a possible homeotic shift in stem cell phenotype. Regional specificity of genes such as *Cdx-1*, *Cdx-2*, and *Tcf-4* appear to influence the morphological features of different regions of the intestinal epithelium, and regulate the proliferation and differentiation of the stem cells.

GASTROINTESTINAL NEOPLASMS ORIGINATE IN STEM CELL POPULATIONS

We can use the development of colorectal carcinoma as a paradigm. The concept of the adenoma–carcinoma sequence, whereby adenomas develop into carcinomas, is now widely-accepted, and most colorectal carcinomas are believed to originate in adenomas. The initial genetic change in the development of most colorectal adenomas is thought to be somatic mutations at the *APC* locus—mutations in APC can be identified in up to 80% of sporadic colorectal carcinomas—and the molecular events associated with these stages are clear: a second hit in the *APC* gene is sufficient to give microadenoma development, at least in FAP. There are two models for adenoma histogenesis, both of which closely involve basic concepts

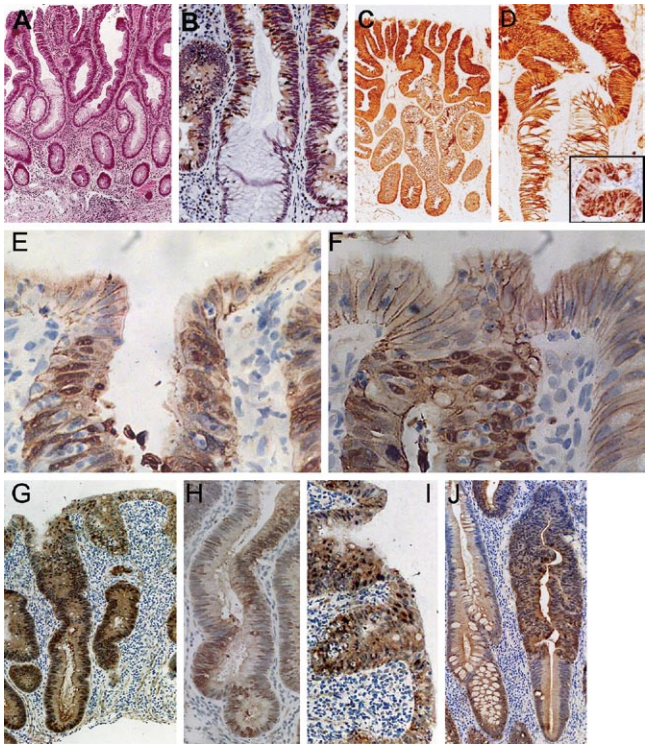


FIGURE 36-11 Contrasting theories for the morphogenesis of adenomas and the part played by stem cells. (A) Hematoxylin- and eosin-stained sections of a small tubular adenoma. Dysplastic epithelium is superficial within the crypts, with histologically normal underlying epithelium. (B) Abrupt transition between dysplastic and normal-appearing epithelial cells at the midpoint of this crypt. Proliferative activity assessed with the Ki-67 antibody, distributed throughout the dysplastic epithelium at the top of the crypts. (C) Nuclear β-catenin is highly expressed and distributed throughout the dysplastic epithelium at the top of the crypts but not in the crypt bases. (D) β-catenin in the nuclei of adenomatous crypts from a tiny tubular adenoma. (E) Nuclear β-catenin extends to the bottom of crypts in early adenomas, including the very bases of the crypts. (F) β-catenin staining in nuclei of budding crypts. (G) Junction between early adenomatous crypts, showing a sharp junction on the surface with accumulation of nuclear β-catenin giving way sharply to membranous staining in the normal surface cells. (H) High-power serial sections, demonstrating the sharp junction between nuclear staining in the adenomatous cells and membranous staining in normal surface epithelial cells. (I) Surface continuity between crypts showing nuclear β-catenin staining. (J) Crypts from a larger adenoma stained for β-catenin, showing invasion of adjacent crypt territories in a top-down fashion. Panels A–D reproduced with permission from Shih and panels E–J reproduced with permission from Preston.

of stem cell biology in the colon: in the "top-down" model, mutant cells appear in the "intracryptal zone" between crypt orifices and, as the clone expands, the cells migrate laterally and downwards, displacing the normal epithelium of adjacent crypts. A modification of this proposal is that a mutant cell in the crypt base, classically the site of the stem cell compartment, migrates to the crypt apex where it expands. These proposals are based on findings in some early non-FAP adenomas, where dysplastic cells were seen exclusively at the orifices and on the luminal surface of colonic crypts; measurement of loss of heterozygosity (LOH) for *APC*, and nucleotide sequence analysis of the mutation cluster region of the *APC* gene carried out on microdissected, well-orientated histological sections of these adenomas showed that half the sample had LOH in the upper portion of the crypts, most with truncating *APC* mutations. Only these superficial cells showed prominent proliferative activity, with nuclear localization of β-catenin indicating an *APC* mutation only in these apical cells (Figure 36-11C). Earlier morphological studies have drawn attention to the same appearances. The top-down morphogenesis has wide implications for established concepts of stem cell biology in the gut. It is clear that most evidence indicates that crypt stem cells are found at the origin of the cell flux, near the crypt base. These proposals, however, seek to either re-establish the stem cell compartment in the intracryptal zone, or make the intracryptal zone a favored locus where stem cells, having acquired a second hit, clonally expand.

An alternative hypothesis—the bottom-up theory—proposes that the earliest lesion is the "unicryptal" or "monocryptal" adenoma, where dysplastic cells occupy an entire single crypt with surrounding normal epitheium, with clonal expansion by crypt fission being the predominant mode of spread of an adenomatous crypt. A stem cell acquires a second hit in *APC*, then it expands stochastically, or more likely because of a selective advantage, to colonize the whole crypt and form a monocryptal adenoma. These lesions are the earliest detecable precursor lesions of adenoma development, and are commonly found in FAP; although they are rare in non-FAP patients they have been described. As would be expected from the experimental data demonstrating the clonal nature of crypts, such monocryptal lesions are indeed clonal. Similar crypt-restricted expansion of mutated stem cells has been well-documented in mice after ENU treatment and in humans heterozygous for the *OAT* gene, where after LOH, initially half, and then the whole crypt is colonized by the progeny of the mutant stem cell. Interestingly, *OAT*+/*OAT*− individuals with FAP show increased rates of stem cell mutation with clustering of mutated crypts. Thus, the mutated clone expands, not by lateral migration, but by "crypt fission," whereby the crypt divides, usually symmetrically at the base (Figure 36-12A) or by budding (Figure 36-12B).

Several studies have shown that fission of adenomatous crypts is the main mode of adenoma progression—predominantly in FAP, where such events are readily evaluated,

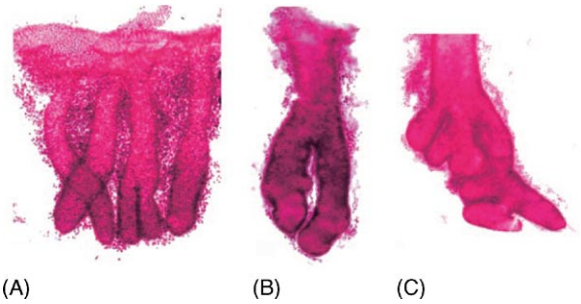


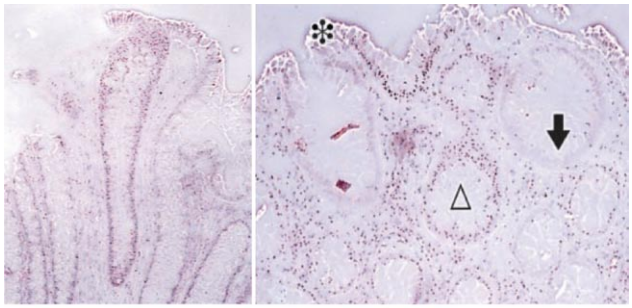
FIGURE 36-12 Microdissected crypts from normal colonic mucosae and adenomas. (A) Symmetrical fission of normal colonic crypts. (B) Isolated adenomatous crypt showing frequent crypt fission with atypical and asymmetrical branching. (C) Another crypt from an adenoma with a bizarre shape, asymmetrical branching, and multiple budding. Reproduced with permission from Preston.

but also in sporadic adenomas. The nonadenomatous mucosa in FAP, with only one *APC* mutation, not only shows a large increase in crypt fission rates, but also expansion of the crypt base stem cell population—a proposed mechanism for increased crypt fission. At the bottom of the crypt, progenitor cells accumulate nuclear β-catenin and express *Tcf* target genes, as a result of *Wnt* stimulation from surrounding basal pericryptal myofibroblasts. In normal crypts, cells that reach the mid-crypt region down-regulate β-catenin/*Tcf*, resulting in cell cycle arrest and differentiation. Cells that bear a β-catenin, or an *APC*, mutation do not respond to signals controlling β-catenin/*Tcf* activity, and these cells continue to behave as crypt progenitor cells in the surface epithelium, generating microadenomas. Boman used computer modeling to suggest that an expansion in the crypt stem cell population explained the putative proliferative abnormality in FAP, namely an upward shift in the proliferative compartment towards the top of the crypt. Simulation of labeling index distribution curves from FAP crypts using a single mechanistic design was able to fit the data from both control and FAP crypts, indicating that the proliferative abnormality does not alter the rate of cell cycle proliferation, differentiation, or apoptosis of proliferating crypt cells. Instead, it suggests an expansion in the crypt stem cell population sufficient to explain the observed proliferative abnormality in FAP. Thus, β-catenin signaling in the colonic crypt controls the number of stem cells. The stem cell population is expanded in FAP crypts, because of a germline APC mutation activating Tcf-4. Any increase in the size of the stem cell population might be expected to result in an increase in the rate of crypt fission. Crypt fission is therefore an essential event in the expansion of mutated clones in adenomas. Although the morphology of this process is distinct, the molecular mechanisms that govern it are far from clear

Aberrant crypt foci (ACF) are clusters of small numbers of abnormal crypts, thought to be a further early step in the development of adenomas, and have been shown to grow by crypt fission, as do hyperplastic polyps. The bottom-up concept does not exclude the possibility that the clone later expands by lateral migration, with downward spread into adjacent crypts, but with the initial lesion the monocryptal adenoma, this model of tumorigenesis is conceptually very different.

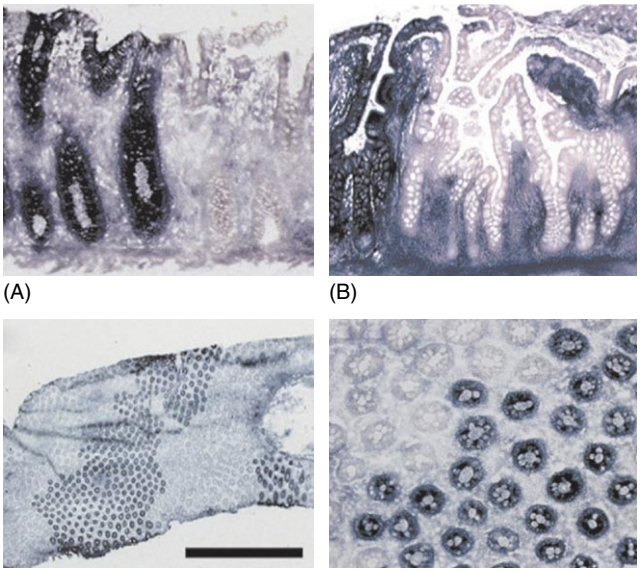
Work supporting the bottom-up spread of colorectal adenomas looked at a number of small (<3 mm) tubular adenomas. Here, nuclear accumulation of β-catenin (Figure 36-11G) was seen, indicating loss of function of one of the genes in the *Wnt* pathway, most likely *APC*, with subsequent translocation of β-catenin to the nucleus. Serial sections (Figure 36-11E, F) showed that the β-catenin nuclear staining extended to the bottom of the crypts, and was present in crypts in the process of crypt fission (Figure 36-11H). β-catenin expression was particularly marked in the nuclei of buds. At the surface, there was a sharp cut-off between the adenomatous cells in the crypt that showed nuclear β-catenin, and those surface cells that did not (Figure 36-11E, F). The adjacent crypts were filled with dysplastic cells containing nuclear β-catenin, which were not confined to the upper portions of the crypts. In larger adenomas, there was unequivocal evidence of surface cells growing down and replacing the epithelium of normal-looking crypts (Figure 36-11J). Crypt fission was rare in normal and non-involved mucosa, and usually began with basal bifurcation at the base of the gland (Figure 36-12A), whereas in adenomas, fission was commonly asymmetrical and disordered, with budding from the superficial and mid-crypt (Figure 36-12B). Multiple fission events were frequently observed in adenomas (Figure 36-12C). The "crypt fission index" (the proportion of crypts in fission) in adenomas has been demonstrated to be significantly greater than that in non-involved mucosa.

It is often stated that adenomas do not display a stem cell architecture: observations on possibly older adenomas have suggested that maximum proliferative activity is found towards the top of the crypts, indicating that migration kinetics are reversed, with cells flowing toward the bottom of the crypt, this is corroborated by the report of increased apoptosis at the bottom of adenomatous crypts. However, more recent experimental data from both the human and mouse demonstrates crypts in early adenomas show similarities to normal crypts in the distribution of their proliferative activity, with an upward expansion of the proliferative compartment towards the surface in adenomas, and maintenance of a stem cell/progenitor hierachy. Tsao investigated the likely microstructure of colorectal adenomas and carcinomas from hereditary nonpolyposis colon cancer, by sampling microsatellite mutations and then using experimental modeling to recreate these measurements; they concluded that the discrete stem cell architecture of normal epithelium is likely to persist in adenomas. This has further been supported



(A) (B)

FIGURE 36–13. Adenomas from an XO–XY mosaic individual with FAP, stained by *in situ* hybridization for a Y-chromosome-specific probe. (A) Clonal monocryptal adenoma. (B) Polyclonal adenoma, with a mixture of XO (arrow) and XY (triangle) crypts. Note that there is a sharp margin between the territories of XO and XY adenomatous crypts (*) at the surface with no evidence of invasion. Reproduced with permission from Preston.



(A) (B) (C) (D)

FIGURE 36-14 Visualization of X-inactivation patches directly by enzyme histochemistry in surgical resections from Sardinian females heterozygous for the G6PD Mediterranean mutation (563 C 3 T), previously shown to have reduced G6PD enzyme activity. Heterozygosity for the G6PD Mediterranean mutation was confirmed by polymerase chain reaction analysis of genomic DNA, followed by MBOII restriction endonuclease. (A) G6PD staining in longitudinal sections of colonic crypts. In individual crypts, all epithelial cells show a similar staining pattern. (B) Longitudinal sections of crypts and villi in small intestinal mucosa stained for G6PD activity. Epithelial cells in individual crypts show a similar staining pattern, but the villous epithelium shows areas of positive and negative staining, confirming its polyclonal derivation. (C) G6PD staining in colonic patches in low power. (D) High-power view of large patches of crypts with irregular patch borders. Bar indicates 2 mm. Reproduced with permission from Novelli.

by Barker who, using lgr5 as a stem cell marker, demonstrated in the mouse that deletion of APC in crypt stem cells allows rapid dysplastic transformation and development of microadenomas. The microadenomas continued to grow and formed macroscopic adenomas in three to five weeks, importantly the stem-cell hierarchy was maintained in the adenomas and deleting APC in non stem cells did not result in sustained adenoma growth—strengthening the concept that it is mutations in stem cells that initiate gastrointestinal cancers.

Examination of the adenomas of an XO–XY individual with FAP showed none exceeded 2.5 mm in diameter. The monocryptal adenomas showed either the XO or the XY genotype, and hence are clonal proliferations, as would be expected from the observation that crypts are clonal (Figure 36-13A). Many microadenomas showed the XY genotype, and none of these early lesions showed a mixture of XO and XY nuclei occupying the same crypt (Figure 36-13B). It was shown that 76% of the microadenomas were polyclonal. There were also sharp boundaries at the surface between adjacent adenomatous crypt territories.

What are the implications of these considerations on our concepts of the single (stem) cell origin or clonality in colorectal adenomas? We have seen that crypts are clonal units; thus, these lesions would be polyclonal because of the mixture of clonal crypts and clonal adenoma—although in this instance, they have different clonal derivation. A study of both sporadic and FAP adenomas, using X-linked restriction fragment length polymorphisms, showed that such lesions were apparently monoclonal in origin. On the other hand, the X-linked patch in the colon is large—and can be in excess of 450 crypts in diameter (Figure 36-14). So, unless an adenoma grows over a patch boundary and involves crypts on either side of that boundary, X-inactivation analysis will always show that such lesions are monoclonal.

On the "bottom-up" proposals, expansion of a clonal monocryptal adenoma by crypt fission would inevitably lead to a monoclonal microadenoma, and thus adenoma. However, as mentioned before, studies on the XO–XY individual, who has a mean patch size of 1.48 crypts, indicated that some 76% of adenomas were polyclonal, supported by observations in Rosa26–Min chimeric mice. Explanations for this are that random collisions occur between independently-transformed neighboring crypts, as there was a high tumor load in this study. Alternatively, active cooperation or clonal interaction is required between dysplastic clones in order for the continued survival and growth of an adenoma.

So, what is happening to the stem cells in the development of these early, monocryptal lesions? Bjerknes showed that adenomatous crypts in FAP contained two stem cell lines, *APC*−/+ and *APC* −/−, and interpreted this to mean that the *APC*−/− cells were expanding stochastically within the same crypt. There is good evidence that such monoclonal conversion occurs as a stochastic

process. It has previously been shown that a quantitative analysis of age-related methylation suggests that crypts are maintained by niches containing multiple stem cells. Random stem cell loss with replacement suggests that all niche lineages except one will become extinct. It is fairly clear that clonal succession is related to tumor progression. Although we have mentioned previously that Shibata has argued for clonal succession occurring in a stem cell niche without mutation, genetic alterations aiding clonal expansion of a mutant clone might confer growth advantages. Several mouse genotypes exist in which crypt morphology—and therefore implicitly stem cell proliferative behavior—is normal, such as APC+/−, TP53+/−, Trp53−/−, Apc+/−, Trp53−/−, MLH1−/0, Apc+/−, Mlh1−/−, and Tgfbr2+/−. However, more subtle alterations, such as an increased incidence of crypt fission that may be dependent upon stem cell behavior, might well be missed.

We have argued that gastrointestinal cancers originate in stem cells; not only are these the only cells that would appear to have a sufficient lifespan required to accumulate all the mutations necessary for malignant transformation, but we now have experimental evidence that only dysplastic transformation of stem cells can drive the formation of adenomas. Recent studies have now shown that a subpopulation of cancer-initiating cells is found in colonic carcinomas. These so-called cancer stem cells were identified by the cell surface marker CD133, and only this CD133 population of cancer cells was able to initiate new tumors in immunodeficent mice. It is proposed, therefore, that cancers themselves are maintained by a population of cancer stem cells, and it is only these cells that are able to migrate and form distant metastases.

Let us now summarize the situation in a normal individual, where stem cells are wild-type (APC+/+), and one APC allele is lost in a single stem cell. This APC+/− stem cell, unless it possesses a growth advantage, could be lost by stochastic ejection from the niche—indeed, it could be argued that this will happen in the over-riding majority of instances, but the APC+/ − cell could survive and the niche will be populated by progeny of this APC+/− cell, which will resemble an FAP crypt. A further hit in APC in this crypt will lead to the formation of a dysplastic crypt—the monocryptal adenoma. Initial growth occurs through crypt fission, with spread into adjacent crypt territories a secondary event, resulting in an aberrant crypt focus, or microadenoma, that in turn expands further to form an established adenoma. We have argued that adenomas are polyclonal, with possible transformation of non-involved crypts by transformed stem cells, and with clonal interaction needed for the survival of the early adenoma. The stem cell hierarchy is maintained in the adenoma, but with an upward expansion in the stem cell compartment, and hugely increased rates of disordered crypt fission. Over time, the adenoma accumulates further mutations in critical genes, resulting in malignant transformation to an adenocarcinoma that, possibly, is also maintained by a cancer stem cell population.

SUMMARY

The cells of the gastrointestinal tract undergo constant renewal, and respond to damage by regeneration and repopulation. Each region of the gastrointestinal tract is morphologically distinct, with its own repertoire of cell types. Although the stem cells are the most important cells of the gastrointestinal tract, responsible for the production of every other cell type in the gastrointestinal mucosa, they have not yet been closely characterized. There is, initially at any rate, a single stem cell in every intestinal crypt or gastric gland that indirectly generates a clone containing further stem cells, transit amplifying and differentiated cells, through the production of committed progenitor cells. This cell also produces new crypts by crypt fission, repairs entire crypts and villi when damaged, and generates gastrointestinal tumors. The stem cell or cells occupy a niche, formed by mesenchymal cells, such as the ISEMFs, and extracellular matrix molecules, which regulate epithelial stem cells through mesenchymal–epithelial crosstalk. The molecular events that regulate the development of the gastrointestinal tract, and epithelial cell turnover in the normal tissue and in formation of carcinomas are beginning to be identified. It is clear that the Wnt/β-catenin signaling pathway and downstream molecules such as APC, Tcf-4, Fkh-6, Cdx-1, and Cdx-2 are vital for normal gastrointestinal stem cell function. We are beginning to identify molecular pathways that determine further proliferation of committed progenitor cells into specific epithelial cell lineages: the Notch–Delta signaling pathways involving Hes1 and Math1 transcription factors regulate differentiation of goblet, Paneth, and enteroendocrine cells in the small intestine. Factors secreted and expressed by the mesenchymal cells (KGF, HGF, etc.) that regulate gastrointestinal mucosal development and epithelial proliferation are rapidly becoming identified. Epithelial and intestinal subepithelial myofibroblast lineages, apparently derived from bone marrow, present the possibility of delivering therapeutic genes to the damaged lamina propria—for example, in diseases that cause fibrosis, such as Crohn's disease—and even repopulating the damaged gut. Finally, we conclude that the colonic stem cell is pivotal in understanding mechanisms of tumorigenesis in the colon. The isolation and characterization of gastrointestinal stem cells is a priority in gut biology.

KEY WORDS

Crypt clonality Intestinal crypts, wherein all cell lineages are originally derived from a single cell during embryonic development.

Gastrointestinal stem cells Founder cells capable of limitless self-renewal throughout the lifetime of their host. Gastrointestinal stem cells are also responsible for the maintenance of tissue homeostasis, by producing daughter cells committed to multipotent differentiation to form all adult lineages within the gastrointestinal tract.

Gastrointestinal stem cell signaling Elucidation of the components involved in molecular pathways, such as Wnt/b-catenin, which regulate cell proliferation and differentiation in the gastrointestinal tract, will provide an insight into the mechanisms of normal epithelial cell turnover and neoplastic development.

Gastrointestinal tumor morphogenesis The mode of dysplastic growth within the gastrointestinal tract is a topic of heated debate, with two main conflicting theories. The first "bottom-up" theory proposes that neoplastic lesions arise in the base of the crypt, the "stem cell zone" and proliferate upwards. The alternative, "top-down" theory states that lesions arise within an "intercryptal" zone, and spread laterally and down into adjacent crypts.

Stem cell plasticity (in the gastrointestinal tract) Evidence suggests that a population of stem cells in the adult bone marrow can engraft within the gastrointestinal tract and form both epithelial and mesenchymal lineages, with an increased propensity in damaged tissue. However, the mechanisms by which bone marrow cells form gastrointestinal lineages are unresolved.

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Part IV

Methods

Chapter 37

Induced Pluripotent Stem Cell Derivation

Junying Yu and James A. Thomson

INTRODUCTION

Derivation of induced pluripotent stem cells directly from differentiated human somatic cells offers an easy and efficient alternative to derive patient-specific pluripotent stem cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Lowry *et al.*, 2008; Park *et al.*, 2008). Through screening genes with enriched expression in human embryonic stem cells, we found that overexpression of *OCT4*, *SOX2*, *NANOG* and *LIN28* alone was sufficient to induce pluripotent stem cells directly from fetal, newborn, and adult human fibroblasts. These induced pluripotent stem cells are morphologically similar to human embryonic stem cells, express typical human embryonic stem cell-specific cell surface antigens and endogenous genes, and are able to differentiate into multiple lineages *in vitro*. More importantly, when injected into immunocompromised mice, they form teratomas containing differentiated derivatives of all three primary germ layers (Yu *et al.*, 2007). In this chapter, we describe the detailed procedure for the derivation of induced pluripotent stem cells, using newborn human foreskin fibroblasts as an example.

MATERIALS

Reagents

- DMEM basal medium (Invitrogen, Cat. No. 11965).
- DMEM/F12 basal medium (Invitrogen, Cat. No. 11330-057).
- IMEM basal medium (Invitrogen, Cat. No. 12440).
- Fetal bovine serum (FBS) (Hyclone, Cat. No. SV30014.03).
- Knockout serum replacer (Invitrogen, Cat. No. 10828-028).
- Non-essential amino acids (Invitrogen, Cat. No. 11140-050).
- GlutaMAX (Invitrogen, Cat. No. 35050-061).
- Sodium pyruvate (Invitrogen, Cat. No. 11360-070).
- β-mercaptoethanol (Sigma, Cat. No. M7522).
- DMSO (Sigma, Cat. No. D-2650).
- Trypsin/EDTA solution (Invitrogen, Cat. No. 25300).
- Collagenase Type IV (Invitrogen, Cat. No. 17104-019).
- PBS without Ca^{2+} and Mg^{2+} (Invitrogen, Cat. No. 14190-250).
- Geneticin (Invitrogen, Cat. No. 10131035).
- Gelatin (Sigma, Cat. No. G1393).
- SuperFect transfection reagent (Qiagen, Cat. No. 301305).
- bFGF (zebrafish) is produced as previously described (Ludwig *et al.*, 2006).
- RNeasy® Mini Kit (Qiagen, Cat. No. 74106).
- QIA shredder (Qiagen, Cat. No. 79656).
- RNase-Free DNase set (Qiagen, Cat. No. 79254).
- SuperScript® III First-Strand Synthesis System (Invitrogen, Cat. No. 18080-051).
- SYBR green QPCR mastermix (Applied Biosystems, Cat. No. 4309155).
- Polybrene (Hexadimethrine bromide) (Sigma, Cat. No. H9268).
- Clorox® regular bleach

Cell Stocks

- 293FT cells (Invitrogen, # R700-07).
- Newborn human foreskin fibroblasts (ATCC, Cat. No. CRL-2097).
- Mouse embryonic fibroblast (MEF) feeder cells are prepared from day 13 CF1 pregnant mouse, and irradiated as previously described (Xu *et al.*, 2004).

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Solutions and Culture Medium

- Polybrene solution (6 mg/ml). Add 60 mg of polybrene to 10 ml water. Filter to sterilize. Aliquot (100 μl each) and store at −20ºC.
- 0.1% gelatin solution. Add 0.5 gram of gelatin powder to 500 ml of water. Autoclave to sterilize.
- 1 mg/ml collagenase solution. Add 50 mg of collagenase type IV to 50 ml of DMEM/F12 basal medium. Filter to sterilize. Store at 4ºC for no longer than one week.
- 293FT cell culture medium. Add 100 ml of heat-inactivated FBS, 10 ml of 200 mM glutamax stock, and 10 ml of 10 mM non-essential amino acid stock to 880 ml of DMEM basal medium to make up the 1 liter volume. Filter to sterilize. Store at 4ºC for no longer than two weeks.
- MEF feeder cell culture medium. Add 100 ml of heat-inactivated FBS and 10 ml of 10 mM non-essential amino acid stock to 890 ml of DMEM basal medium to make up the 1 liter volume. Filter to sterilize. Store at 4ºC for no longer than two weeks.
- Human ES cell culture medium without bFGF. Add 200 ml of Knockout Serum Replacer, 5 ml of 200 mM glutamax stock, 10 ml of 10 mM non-essential amino acid stock and 7 μl of β-mercaptoethanol to 785 ml of DMEM/F12 basal medium to make up the 1 liter volume. Filter to sterilize. Store at 4ºC for no longer than two weeks.
- Human ES cell culture medium with bFGF. Add 100 μg zebrafish bFGF to 1 liter of human ES cell culture medium without bFGF to obtain a final bFGF concentration of 100 ng/ml. Filter to sterilize. Store at 4ºC for no longer than two weeks.

Supplies and Equipment

- T75 tissue culture treated flasks (Fisher, Cat. No. 10-126-7).
- T175 tissue culture treated flasks (Fisher, Cat. No. 12-565-54).
- 10 cm tissue culture treated dishes (Fisher, Cat. No. 08-772E).
- 6-well tissue culture treated plates (Fisher, Cat. No. 14-832-11).
- 48-well tissue culture treated plates (Fisher, Cat. No. 12-565-322).
- Cell culture filters–50 ml (Fisher, Cat. No. SE1M179M6).
- Cell culture filters–150 ml (Fisher, Cat. No. SCGPU01RE).
- Cell culture filters–250 ml (Fisher, Cat. No. SCGPU02RE).
- Cell culture filters–500 ml (Fisher, Cat. No. SCGPU05RE).
- Cell culture filters–1000 ml (Fisher, Cat. No. SCGPU11RE).
- 115 ml low-protein binding cellulose acetate filters (0.45 μM) (Corning, Cat. No. 430945).
- 15 ml conical tubes (Fisher, Cat. No. 05-538-53D).
- 50 ml conical tubes (Fisher, Cat. No. 05-538-55).
- Incubators: all cells are maintained in a humidified incubator in a 5% CO2/95% air atmosphere at 37ºC.
- Optima™ L-80 XP ultracentrifuge (Beckman, PN # 392051).
- 45Ti rotor for ultracentrifuge (Beckman, PN # 339160).
- 70 ml centrifugation bottles (Beckman, PN # 355622).
- Horizontal shaker (Fisher, Cat. No. 11+67152Q).
- Thermocyler (Applied Biosystems, Cat. No. 4375786).
- Real-time PCR system (Applied Biosystems, Cat. No. 4351106).
- 96-well PCR plate (Fisher, Cat. No. NC9726586).
- Optimal Adhesive Film (Fisher, Cat. No. NC9745301).

Plasmids

- pSIN4-EF2-EGFP-IRES-Puro
- pSIN4-EF2-OCT4-IRES-Puro
- pSIN4-EF2-SOX2-IRES-Puro
- pSIN4-EF2-NANOG-IRES-Puro
- pSIN4-EF2-LIN28-IRES-Puro
- psPAX2
- pMD.G

PREPARING TRANSGENE-EXPRESSING LENTIVIRUS

Packaging Lentivirus

1. Maintain 293FT cells in 293FT cell culture medium supplemented with 500 μg/ml geneticin according to the product manual (Invitrogen).
2. For the lentiviral packaging, 293FT cells are expanded in T175 tissue culture treated flasks in 293FT cell culture medium supplemented with 500 μg/ml geneticin. The number of T175 flasks needed is approximately twice the number of constructs to be packaged. Gelatin-coating of T175 flasks is not needed.
3. Day 1 (~11 am–12 pm): when 293FT cells reach approximately 80–90% confluency, remove culture medium, and wash cells with 8 ml/T175 flask of PBS without Ca^{2+} and Mg^{2+}.
4. Add 6 ml/T175 flask of Trypsin/EDTA solution, and incubate at room temperature for three to five minutes.
5. Add 12 ml/T175 flask of 293FT cell culture medium to neutralize the trypsin activity.
6. Transfer 293 FT cells to 50 ml conical tubes, and pellet cells by centrifugation at 200 × g for five minutes.

7. Remove supernatant, and gently resuspend 293FT cells in 293FT cell culture medium without geneticin (approximately 30 ml for each T175 flask of 293FT cells). The cell density is usually between $1 - 1.5 \times 10^6$ cells/ml.
8. For each construct, prepare 6 × T75 tissue culture treated flasks. Gelatin-coating of T75 flasks is not needed.
9. Aliquot 10 ml of 293FT cell suspension to each T75 flask.
10. Prepare transfection mixture as steps 11 to 14 (Table 37-1).
11. Centrifuge plasmids pMD.G, psPAX2 and transgene-expressing pSIN vectors at maximum speed (~20 000 × g) for 10 minutes, to remove debris and possible bacterial contamination. The concentration of all plasmids should be at least 0.5 μg/μl, to avoid dilution when they are combined with transfection reagents.
12. Mix the corresponding amount of each plasmid in room temperature serum-free IMDM basal medium, as in Table 37-1. Prepare enough plasmids in 15 ml conical tubes for 6 × T75 flasks for each transgene-expressing construct.
13. Add the corresponding amount of Superfect to the DNA solution, and mix with brief vortexing.
14. Briefly spin down to collect liquid at the bottom of the tube, and incubate the DNA/Superfect mixture at room temperature for 10 minutes.

Transfection of 293FT Cells

1. To transfect 293FT cells, add fresh 293 FT cell culture medium to the DNA/Superfect mixture (~3.4 ml for 6 × T75 flasks), and mix by pipetting up and down three times. Avoid bubble formation.
2. Add DNA/Superfect mixture dropwise to the 293FT cell suspension (~1 ml/T75 flask).
3. Gently mix 293FT cells, and incubate at 37ºC overnight.
4. Day 2 (~9 am–10 am): replace the transfection medium with fresh 293FT cell culture medium, supplemented with 1 mM sodium pyruvate (~8 ml/T75 flask).

TABLE 37-1 Transfection of 293FT Cells

	per T75 flask	6 x T75 flasks
pMD.G	5 μg	30 μg
psPAX2	10 μg	60 μg
pSIN vector	5 μg	30 μg
Superfect	40 μl	240 μl
IMDM	400 μl	2.4 ml

5. Day 4 (~9 am): collect lentivirus (~68 to 72 hours post-transfection) by pouring the supernatant from 6 × T75 flasks for each transgene-expressing construct directly to a 50 ml conical tube.
6. Remove cell debris by centrifugation at 1000 × g for five minutes.
7. Filter the supernatant through 115 ml low-protein binding cellulose acetate filters (0.45 μM).
8. Concentrate lentivirus through ultracentrifugation of the supernatant in 70 ml sterilized tubes at 50 000 × g for 2.5 hours at 4ºC. The 70 ml centrifugation tubes can be sterilized by Clorox® regular bleach, followed by three washes with sterile water.
9. Carefully remove the supernatant without disturbing the pellet.
10. Add ~500 μl of PBS without Ca^{2+} and Mg^{2+}, and resuspend the pellet by gently shaking the centrifuge tubes at 4ºC for about three to six hours on a horizontal rocker.
11. Transfer the resuspended lentivirus to 2 ml eppendorf tubes pre-chilled at 4ºC.
12. Remove any residual debris from the lentiviral preparations by centrifugation at 1000 × g for 10 minutes at 4ºC.
13. Aliquot (~50 μl each) the lentivirus-containing supernatant to 0.5 ml eppendorf tubes pre-chilled at −20ºC, and store at −80ºC.

Titering Lentivirus

Determine the Titer for EGFP-expressing Lentivirus Through Transduction

1. A 6-well tissue culture treated plate is coated with gelatin by adding 1.5 ml/well of 0.1% sterile gelatin solution, and incubated at 37ºC for at least one hour. Remove the gelatin solution immediately prior to use.
2. Grow 1 × T75 flask of 293FT cells in 293FT cell culture medium supplemented with 500 μg/ml geneticin.
3. When 293FT cells reach approximately 80–90% confluency, remove culture medium, and wash cells with 6 ml of PBS without Ca^{2+} and Mg^{2+}.
4. Add 3 ml of Trypsin/EDTA solution to the T75 flask, and incubate at room temperature for three to five minutes.
5. Add 6 ml of 293FT cell culture medium to the T75 flask to neutralize the trypsin activity.
6. Transfer 293 FT cells to a 15 ml conical tube, and pellet cells by centrifugation at 200 × g for five minutes.
7. Remove the supernatant, and gently resuspend 293FT cells in 10 ml of 293FT cell culture medium without geneticin.
8. Determine and adjust the cell density to about 1×10^6 cells/ml.
9. Add 1 ml/well of 293FT cells to the gelatin-coated 6-well plate (1×10^6 cells /well).

10. Add 1 μl of polybrene (6 mg/ml stock) to each well.
11. Add the following amount of the concentrated EGFP-expressing lentivirus to each well (μl): 0, 1, 2, 5, 10, 20.
12. Mix 293FT cells and incubate at 37ºC overnight.
13. The next day, replace the transduction mixture with 3 ml of fresh 293FT cell culture medium.
14. After 48–72 hours post-transduction, determine the percentage of EGFP positive cells in each well using flow cytometry analysis.
15. Use the percentages of non-lentivirus-saturating wells to calculate the titer for the EGFP-expressing lentivirus. For example, if the percentage of the 2 μl-well is "a", the percentage of the 5 μl-well is "b", and both "a" and "b" are less than 90%, the titer for the EGFP-expressing lentivirus is the following: $[(a \times 10^6/2 + b \times 10^6/5)/2 \times 10^3$ viral particles (vp)/ml, i.e., $[(a/2 + b/5)/2] \times 10^9$ vp/ml. The titer for the EGFP-expressing lentivirus is generally greater than 1×10^8 vp/ml using the packaging protocol above.

Determine the Titers for OCT4, SOX2, NANOG and LIN28-expressing Lentivirus through Real-time RT-PCR

1. Extract total RNA from 10 μl of the concentrated lentivirus expressing EGFP (as a relative standard), OCT4, SOX2, NANOG, and LIN28, as described in the RNeasy® Mini Kit (Qiagen). To remove DNA contamination, on-column DNA digestion is carried out with RNase-free Dnase, as suggested in the RNeasy® Mini Kit. To reduce experimental errors, RNA is extracted from triplicates of each transgene-expressing lentivirus.
2. Elute RNA in 50 μl of sterile water.
3. Take 8 μl of each eluate for reverse transcription (RT) reactions with random hexamers as described in the Superscript III First-Strand Synthesis System (Invitrogen) (Table 37-2).

TABLE 37-2 First Strand cDNA Synthesis

Reagents	Volume (μl)
RNA	8
Random hexamers (50 ng/μl)	1
Incubate at 70ºC for 10 minutes Immediately transfer to ice for 2 minutes	
10× RT buffer	2
25 mM $MgCl_2$	4
DTT (0.1 M)	2
dNTPs (10 mM)	1
RNaseOUT (40 U/μl)	1
Superscript RT III (200 U/μl)	1
Mix and incubate at room temperature for 10 minutes	
Incubate at 50ºC for 50 minutes	
Incubate at 85ºC for 5 minutes to inactivate the enzyme	
Chill on ice and proceed to real-time PCR or store at −20ºC	

1. Real-time PCR is carried out with the following primer set (100 bp): Titer-F (5′ gacaggcccgaaggaatag 3′) and Titer-R (5′ actgccatttgtggcgatac 3′).
2. 10 μl of cDNAs from EGFP-expressing lentivirus (triplicate) is diluted with the following dilution factors: 1, 2^1, 2^2, 2^3, 2^4, 2^5, 2^6, 2^7, which are used as a relative standard for real-time PCR.
3. cDNAs from the OCT4, SOX2, NANOG and LIN28-expressing lentivirus are diluted eight-fold, e.g., 5 μl of cDNA to 35 μl of sterile water.
4. Real-time PCR is carried out with SYBR green QPCR Master Mix (Applied Biosystems) (Table 37-3).

TABLE 37-3 Real-time PCR Set-up

Components	Volume (μl)
PCR Master Mix (2×)	12.5
20 μM Titer-F	0.25
20 μM Titer-R	0.25
Template	1
Water	11
Total	25
95ºC: 10′ 40 × [95ºC: 30″, 60ºC: 1′] 72ºC: 3′	

1. Calculate the titers for the OCT4, SOX2, NANOG and LIN28-expressing lentivirus as the following:
   a. Plot $-\log_2$ (dilution factor) (y) against $Ct_{ave.}$ (x), using results from the EGFP-expressing lentivirus standard in Microsoft Excel. Ct_{ave} is the average of triplicate Ct values for the EGFP-expressing lentivirus. Add the linear trendline to the data, and obtain equation y = a*x + b.
   b. Use the following equation to calculate the titer for each transgene-expressing lentivirus: $[(2^{-(a \bullet Ct1+b)} + 2^{-(a \bullet Ct2+b)} + 2^{-(a \bullet Ct3+b)})/3] * 8 *$ titer (EGFP)

vp/ml. The titer (EGFP) refers to the infectious titer determined through transduction of 293FT cells as shown above.

REPROGRAMMING HUMAN FORESKIN FIBROBLASTS

Culturing Human Foreskin Fibroblasts

1. Perform a quick thaw of a vial of frozen human foreskin fibroblasts (ATCC, Cat. No. CRL-2097) in a 37ºC water bath. To avoid contamination, do not immerse the vial above the level of the cap.
2. When only a small crystal of ice remains in the tube, take the vial out of the water bath, and wipe dry.
3. Decontaminate the vial exterior with 70% ethanol.
4. Transfer the content of the vial to a 15 ml conical tube.
5. Slowly add 10 ml pre-warmed 293FT cell culture medium to the tube dropwise to reduce osmotic shock.
6. Pellet the cells by centrifugation at 200 × g for five minutes.
7. Remove the supernatant, and gently resuspend the foreskin fibroblasts in 10 ml 293FT cell culture medium.
8. Transfer the cells to a T75 tissue culture treated flask. No gelatin coating is needed.
9. Replace the medium the next day, and thereafter every three days.
10. When fibroblasts reach around 90% confluency, split the cells at a ratio of 1:6 according to the product manual (ATCC). Foreskin fibroblasts can be frozen down with 95% 293FT cell culture medium supplemented with 5% DMSO as suggested in the product manual.

Preparing Mouse Embryonic Fibroblast Dishes and Plates

1. 10 cm tissue culture treated dishes, 6-well and 48-well tissue culture treated plates are coated with gelatin by adding 0.1% sterile gelatin solution (6 ml/10 cm dish, 1.5 ml/well of 6-well plates and 0.5 ml/well of 48-well plates), and incubated at 37ºC for at least 1 hour. Remove the gelatin solution immediately prior to use.
2. Mouse embryonic fibroblast (MEF) feeder cells are prepared from day 13 CF1 pregnant mouse and irradiated as previously described (Xu *et al.*, 2004).
3. Irradiated MEF are resuspended in MEF feeder cell culture medium at a density of 0.75×10^5 cells/ml.
4. Add the following amount of MEF to each gelatin-coated culture dish or plate: 15 ml/10 cm dish, 2.5 ml/well of 6-well plates and 0.3 ml/well of 48-well plates.
5. Incubate at 37ºC overnight. Remove the MEF feeder cell culture medium immediately prior to use.
6. The MEF dishes or plates can be used to support reprogramming culture or culture of human ES cells and human induced pluripotent stem (iPS) cells for up to two weeks.

Preparing Conditioned Medium

1. T75 flasks are coated with gelatin by adding 6 ml of 0.1% sterile gelatin solution, and incubated at 37ºC for at least one hour. Remove the gelatin solution immediately prior to use.
2. Irradiated MEF are resuspended in MEF feeder cell culture medium at a density of 2.12×10^5 cells/ml.
3. Add 20 ml of irradiated MEF to each gelatin-coated T75 flask, and incubate at 37ºC overnight.
4. Remove the MEF feeder cell culture medium the next day, and wash the cells once with 8 ml/ T75 flask of PBS without Ca^{2+} and Mg^{2+}.
5. Add 20 ml fresh human ES cell culture medium without bFGF to each T75 MEF flask and incubate at 37ºC for about 24 hours.
6. Collect the conditioned human ES cell culture medium without bFGF (CM). The CM can be stored at −80ºC. To be used in reprogramming culture, the CM is sterilized by filtration following the addition of bFGF at a final concentration of 100 ng/ml, which can be stored at 4ºC for up to two weeks.
7. Repeat step 5 and 6 for five days with 20 ml/ T75 flask of human ES cell culture medium without bFGF, and eight additional days with 15 ml/ T75 flask of human ES cell culture medium without bFGF.

Reprogramming Human Foreskin Fibroblasts

1. A 6-well tissue culture treated plate is coated with gelatin by adding 1.5 ml/well of 0.1% sterile gelatin solution, and incubated at 37ºC for at least one hour. Remove the gelatin solution immediately prior to use.
2. Prepare 12 × 10 cm MEF dishes.
3. Grow human foreskin fibroblasts in T75 flasks in 293FT cell culture medium.
4. When human foreskin fibroblasts reach around 80–90% confluency, remove culture medium, and wash cells with 6 ml of PBS without Ca^{2+} and Mg^{2+}.
5. Add 3 ml of Trypsin/EDTA solution to the T75 flask, and incubate at 37ºC for three to five minutes.
6. Add 6 ml of 293FT cell culture medium to the T75 flask to neutralize the trypsin activity.
7. Transfer the individualized foreskin fibroblasts to a 15 ml conical tube, and pellet cells by centrifugation at 200 × g for five minutes.
8. Remove the supernatant, and gently resuspend 293FT cells in 4 ml of 293FT cell culture medium.
9. Determine and adjust the cell density to about 1×10^6 cells/ml.

10. Add 2 ml/well of fresh 293FT cell culture medium to two wells of the gelatin-coated 6-well plate.
11. Add 0.15 ml/well of the resuspended human foreskin fibroblasts to the two wells prefilled with fresh 293FT cell culture medium ($\sim 1.5 \times 10^5$ cells/well), and incubate at 37ºC.
12. The next day, when fibroblasts reach ~70–80% confluency, perform the lentiviral transduction by adding 2.5 μl of polybrene (6 mg/ml stock) and the following amount of lentivirus: OCT4 ($\sim 2 \times 10^6$ vp), SOX2 ($\sim 8 \times 10^5$ vp), NANOG ($\sim 2 \times 10^6$ vp), and LIN28 ($\sim 4 \times 10^5$ vp) to one well. The other well of fibroblasts is used as a non-transduced control.
13. Incubate the fibroblasts with the lentivirus overnight at 37ºC.
14. The next day (day 1 post-transduction), remove the lentivirus-containing culture medium, and wash cells with 2 ml/well of PBS without Ca^{2+} and Mg^{2+}.
15. Add 1 ml/well of Trypsin/EDTA solution to the fibroblasts, and incubate at 37ºC for three to five minutes.
16. Add 5 ml/well of fresh 293FT cell culture medium to neutralize the trypsin activity.
17. Prepare the 12 × 10 cm MEF dishes by replacing the MEF feeder cell culture medium with 15 ml/dish of fresh 293FT cell culture medium.
18. Aliquot 1 ml/dish of the individualized control and lentivirus-transduced fibroblasts to the 10 cm MEF dishes, and incubate at 37ºC.
19. Two days following fibroblast transfer to the 10 cm MEF dishes (day 3 post-transduction), the 293FT cell culture medium is replaced with 10 ml/dish of human ES cell culture medium with bFGF.
20. The culture medium is exchanged every two days.
21. When a significant number of MEF die off, which usually occurs around day 10 post-transduction, the human ES cell culture medium with bFGF is replaced with 10 ml/dish of the CM supplemented with bFGF.
22. The CM with bFGF is exchanged every two days.
23. The earliest human foreskin iPS cell colonies are generally visible around day 14 post-transduction. Between day 20 and 25 post-transduction, on average, there will be around 5 to 15 iPS cell colonies on each 10 cm dish, about one third of which can be picked for expansion (see below). The morphology of various iPS cell colonies and a typical non-iPS-cell colony at day 22 post-transduction are shown in Figure 37-1.

Picking iPS Cell Clones

1. Prepare a 48-well MEF plate prior to iPS cell colony picking.
2. Immediately before iPS cell colony picking, add 0.5 ml/well of human ES cell culture medium with bFGF to an empty 48-well tissue culture treated plate.

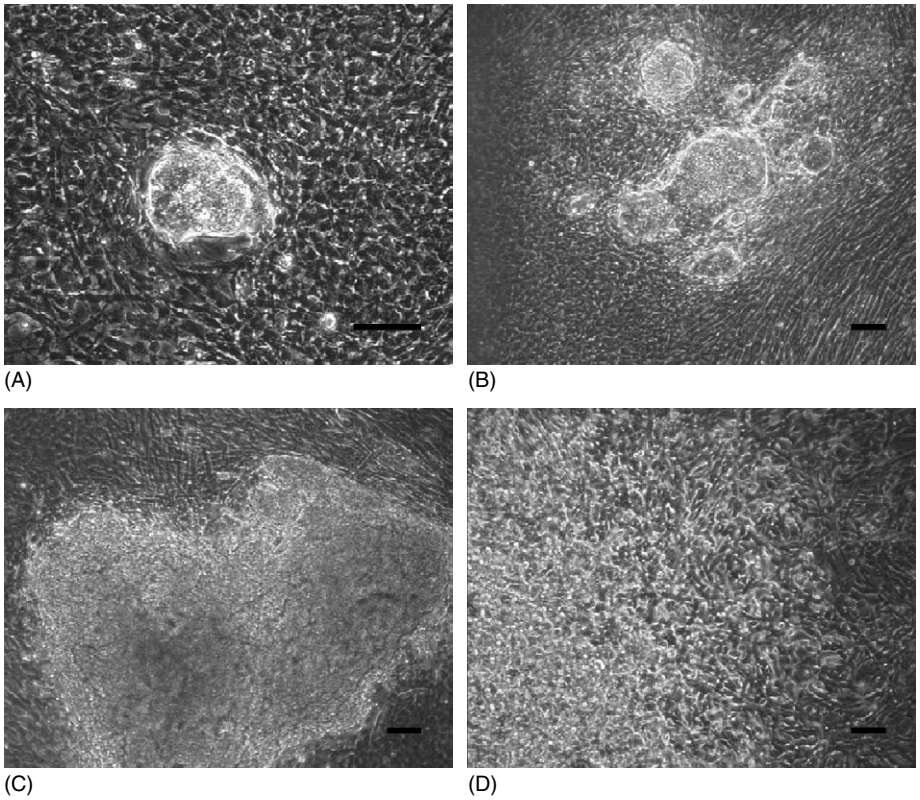


FIGURE 37-1 Morphology of typical iPS cell and non-iPS-cell colonies on day 22 post-transduction of human foreskin fibroblasts. (A) A single late-arising iPS cell colony. (B) A cluster of iPS cell colonies, which most likely originates from a single transduced fibroblast. (C) An overgrown iPS cell colony. (D) A non-iPS-cell colony. Scale bar: 0.1 mm.

3. At day 20 to 25 post-transduction, use a 20 μl pipette (set to 10 μl) to pick each iPS cell colony to each well of the 48-well plate prefilled with human ES cell culture medium with bFGF under a dissection microscope.
4. Use a 1000 μl pipette (set to 600 μl) to mechanically break up the picked iPS cell colonies by pipetting up and down three times.
5. Remove the MEF feeder cell culture medium from the 48-well MEF plate.
6. Transfer the picked iPS cell colonies to the 48-well MEF plate, and incubate at 37ºC.
7. After two days of incubation, replace the culture medium with fresh human ES cell culture medium with bFGF, followed by daily exchange of culture medium.

Expanding Induced Pluripotent Stem Cell Clones

1. Prepare the appropriate number of 6-well MEF plates.
2. At day 7 to 10 following colony picking when iPS cell clones are large enough to split, remove culture medium from the iPS cells.
3. Add 0.5 ml/well of collagenase solution (1 mg/ml) to the 48-well plate.
4. Incubate at 37ºC for 6–8 minutes. iPS cell colonies should curl up around the edge, but do not come off the plate.
5. Remove the collagenase solution.
6. Wash the iPS cells three times with 0.5 ml/well of DMEM/F12 basal medium.
7. Add 0.5 ml/well of fresh human ES cell culture medium with bFGF.
8. Remove MEF feeder cell culture medium from the 6-well MEF plates.
9. Add 2 ml/well of fresh human ES cell culture medium with bFGF to the 6-well MEF plates.
10. Use a 1000 μl pipette (set to 600 μl) to mechanically break up the collagenase-treated iPS cells by pipetting. DO NOT BREAK UP THE iPS CELL COLONIES TOO MUCH AT THIS STEP.
11. Transfer each iPS cell clones to each well of 6-well MEF plates, and incubate at 37ºC.
12. After two days of incubation, replace the culture medium with fresh human ES cell culture medium with bFGF, followed by daily exchange of culture medium.
13. From this step on, the culture and characterization of human iPS cells can be carried out as that for normal human ES cells as previously described (Xu *et al.*, 2004).

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KEY WORDS

Differentiation Process of specialization wherein a cell, through changes in gene expression, acquires the correct shape, polarity, and orientation with respect to its neighbors and the extracellular matrix, and an appropriate set of organelles and proteins to enable it to carry out metabolic, signaling, transport, contractile, structural, or other specific functions in a particular tissue.

Fibroblast A cell in connective tissue that produces collagen and other fibers.

Induced pluripotent stem cells A type of pluripotent stem cell artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a "forced" expression of certain genes.

Lentiviral vector A type of retrovirus that can infect both dividing and nondividing cells, because their preintegration complex (virus "shell") can get through the intact membrane of the nucleus of the target cell.

Pluripotent Able to give rise to differentiated cells of all three germ layers. Cells of the inner cell mass and ES cells are pluripotent.

Teratoma A tumor composed of tissues of multiple germ layer lineages.

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Chapter 38

Characteristics and Characterization of Human Pluripotent Stem Cells

Anne G. Bang and Melissa K. Carpenter

INTRODUCTION

Pluripotent stem cells are defined as cells that have the capacity to differentiate into all three germ layers, specifically: ectoderm; mesoderm; and endoderm, which can give rise to all cell types of the body. These cell populations can be derived from embryonic tissue sources such as the inner cell mass, the epiblast, and primordial germ cells or they can be induced by reprogramming using somatic cell nuclear transfer (SCNT), or the introduction of pluripotency genes into somatic cells. These different pluripotent stem cell populations have similarities and differences, as discussed below.

Embryonic Cell Sources

Embryonic Carcinoma Cells

Embryonic carcinoma (EC) cells were the first pluripotent cells identified. These cells were derived from malignant germ cell tumors called teratocarcinomas. These tumors are characterized by the presence of cell types from all three germ layers, as well as undifferentiated cells. Mouse EC cells were initially derived from testicular tumors, and were utilized as *in vitro* models of development. Human EC cells were subsequently derived, and these cells show surface marker expression that is different from mouse EC cells. For example, mouse EC cells express SSEA-1, while human EC cells express SSEA3, SSEA-4, TRA-1-60, and TRA-1-81. It remains unclear whether these differences in surface marker expression are correlated with functional differences between the cell lines. Another difference between mouse and human cells is their cytogenetic stability. While mouse EC cells are generally euploid, human EC cells are aneuploid, which limits their utility.

Embryonic Stem Cells

Mouse embryonic stem cells (ESCs) were first isolated, over 20 years ago, by removing the inner cell mass (ICM) from mouse pre-implantation embryos and serially passaging these cells on inactivated mouse feeder layers. The resulting cell lines showed unrestricted proliferative potential, and were pluripotent. These ESCs differentiated into derivatives of all three germ layers and, on injection into blastocysts, they contributed to all the tissue types in the resulting chimera. Because these cell lines can be genetically modified, they have been largely used for the generation of transgenic or knockout mice, but they also provide a unique tool for studying the early development of the embryo.

The first human ESC (hESC) lines were derived by Thomson *et al.* from the ICM of surplus human embryos resulting from assisted reproductive procedures. Since the first report in 1998, hESC lines have been derived in multiple laboratories throughout the world (see, for example, the NIH registry website at http://escr.nih.gov).

To date, ESCs have been derived from mouse, rat, and non-human primate and human embryos. While ESCs from these different species share many characteristics, there are also some differences. The defining features of ESCs include: derivation from preimplantation embryos; pluripotency; capacity for prolonged proliferation and self-renewal; a normal euploid karyotype; and the expression of distinctive markers. Importantly, not only the derivation methods, but also the culture conditions and handling of ESC lines determine their successful maintenance. Existing hESC lines were derived in different laboratories using different procedures, which may result in cell lines with different qualities. Although some of the lines have been characterized extensively, most cell lines have not been available for long enough to allow full characterization; therefore, further study will be required to definitively determine their differences and similarities.

Similar to EC cells, mouse, rat, and human ESCs show species specific differences in surface marker expression, but all express characteristic markers of pluripotency, including OCT4, SOX2, and NANOG (Table 38-1). Although all three species express common pluripotency markers, the rodent cell lines appear to be fundamentally different from

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TABLE 38-1 Comparison of Mouse, Rat, and Human Pluripotent Cells

	Mouse EC	Human EC	Mouse ES	Rat ES	Human ES	Mouse epiblast	Mouse EGCs	EGCs	Mouse iPSCs	Human iPSCs
Alkaline Phosphatase	+	+	+	+	+	−	+	+	+	−
SSEA-1	+	−	+	+	−	+	+	+	+	−
SSEA-3	−	+	−	−	+	−	−	+	−	+
SSEA-4	−	+	−	−	+	−	−	+	−	+
TRA-1-60	−	+	−	+	+	−	−	+	−	+
TRA-1-81	−	+	−	+	+	−	−	+	−	+
OCT 3/4	+	+	+	+	+	+	+	+	+	+
NANOG	+	+	+	+	+	+	+	+	+	+
SOX 2	+	+	+	+	+	+	+	N/A	+	+
REX 1	N/A	+	+	+	+	−	N/A	N/A	+	+
TERT	+	+	+	N/A	+	N/A	+	+	+	+
FGF4	+	+	+	+	+	N/A	N/A	N/A	+	+
UTF-1	+	+	+	N/A	+	N/A	N/A	N/A	+	N/A
CRIPTO	+	+	+	N/A	+	+	+	N/A	+	+

the human lines in the signaling pathways responsible for self-renewal. Mouse and rat ESC self-renewal is regulated via the LIF/STAT3 signaling pathway, while self-renewal in human ESCs appears to be regulated via the bFGF signaling pathway. It is unclear whether these differences are indicative of other fundamental differences between species.

ESCs can also be derived from parthenogenetic embryos. In this case, oocytes arrested at the second meiotic metaphase are activated in the presence of cytochalasin, such that extrusion of the second polar body is prevented. The resulting parthenogenetic zygote develops into a blastocyst from which diploid ESC lines that are genetically identical to the egg donor can be isolated (reviewed in Cibelli *et al.*, 2006). Using this or similar processes, ESCs have been derived from mouse, non-human primate, and human parthenogenetic blastocysts. Although this work has been very intriguing, several groups have found abnormalities in these ESCs which may impact their ability to function normally. More recently, it has been shown that ESCs derived from mouse parthenogenetic embryos can generate teratomas, and can contribute to chimeric mice after injection into the blastocyst, although no germline transmission was observed. Thus, ESCs derived from parthenogenetic embryos are capable of differentiation to many lineages; however, it is still unclear whether homozygosity in some regions of the genome and/or lack of paternal imprinting might disrupt cell function.

Embryonic Germ Cells

Primordial germ cells have been shown to be capable of generating ESC-like pluripotent stem cells called embryonic germ cells (EGCs). Mouse EGCs show the fundamental characteristics of pluripotent cells; they can generate teratomas, and can contribute to chimeric mice after injection into a blastocyst. Human EGCs were isolated in 1998, but to date, the proliferation of these cells appears quite limited (Turnpenny *et al.*, 2006). These cells can differentiate into various cell types *in vitro*, but teratoma formation has not been demonstrated. More recently, pluripotent stem cells have been derived from both mouse and human testis. It remains unclear whether these cells represent an endogenous cell population, or are a product of the *in vitro* culture system.

Epiblast Cells

As the early embryo develops, the cells transition from the totipotent zygote to the pluripotent ICM and epiblast. Recently, pluripotent cells have also been isolated from the mouse epiblast. These cells show expression of the pluripotency transcription factors OCT4, SOX2, and NANOG, differentiate *in vitro*, and generate teratomas, but do not contribute to chimeras. Similar to ESCs, these cells show extensive self-renewal; however, they require the presence

of FGF and activin for proliferation, rather than LIF. Therefore, it has been speculated that human ESCs are more similar to mouse epiblast cells than mouse ESCs, and therefore actually represent human epiblast cells (Rossant, 2008). As these different cell populations are more extensively characterized, these distinctions should become clear.

Reprogrammed Cells

Pluripotency can be achieved by reprogramming. Reprogramming induces the dedifferentiation of somatic cells, which is thought to occur through the erasure of epigenetic signatures of the cell, resulting in an increase in potency. Most of the work in the reprogramming field has been done using nuclear transfer techniques (reviewed in Gurdon and Melton, 2008; Jaenisch and Young, 2008). More recently, several groups have demonstrated that reprogramming can be induced by the introduction of pluripotency genes. Successful reprogramming of somatic cells essentially "dedifferentiates" these cells to a pluripotent state, and allows the subsequent derivation of pluripotent cell lines. The generation of pluripotent cells using reprogramming allows generation of cell lines which are genetically identical to the donor.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) takes advantage of a unique property of the oocyte cytoplasm that allows somatic nuclei to be reprogrammed to a pluripotent state. In this case, the nucleus of a somatic cell is transferred into an enucleated oocyte. The somatic nucleus is then reprogrammed, and partial development to the ICM stage can occur in culture, followed by either transplantation into a prepared uterus in order to generate cloned animals, or harvesting the ICM to generate ESC lines (reviewed in Yang *et al.*, 2007; Gurdon and Melton, 2008). In 2005, a group in South Korea reported the generation of human ESCs from patient specific blastocysts created using SCNT. Unfortunately, this work was later shown to be fraudulent, and to date, the generation of human ESC lines using SCNT has not been reported.

Induced Pluripotent Cells

More recently, it has been shown that pluripotency can be achieved by reprogramming adult and fetal somatic cells by the introduction of four genes, OCT4, SOX2, KLF4, c-MYC or OCT4, SOX2, NANOG, and Lin28, into mouse and human fibroblasts. The resulting induced pluripotent stem cells (iPSCs) show many of the characteristics of ESCs, including similar marker expression (Table 38-1), the ability to differentiate into all three germ layers and, in the case of mouse cells, to generate germline chimeras.

CHARACTERIZATION OF PLURIPOTENT CELLS

Markers

Identification of reliable markers for the characterization of pluripotent cells is of great importance in order to exploit their potential. To date, human pluripotent cell lines have been characterized using markers previously used to characterize other pluripotent stem cell populations, such as human embryonal carcinoma (EC) cells and mouse ESCs. The comparison of expression profiles of the different pluripotent cell populations presents similarities and dissimilarities between species and cell type. As shown in Table 38-1, expression of surface markers varies between these different cell populations. Similar to mouse ESCs, the hESCs express alkaline phosphatase-related antigens. The undifferentiated hESCs express the globoseries glycolipid antigens designated stage-specific embryonic antigen (SSEA-3 and SSEA-4), which are expressed by human EC cells, but not by mouse ESCs. In contrast to the mouse ESCs, the undifferentiated hESCs lack expression of a lactoseries oligosaccharide antigen SSEA-1. The keratin sulfate-related antigens, TRA-1-60 and TRA-1-81, expressed by human EC cells are also expressed by hESCs. Although the expression of surface markers has been a very useful tool in characterizing the different pluripotent cell populations, none of these markers has been functionally associated with pluripotency. For instance, the depletion of SSEA-4 from human ESCs does not impact the ability to proliferate or differentiate into all three germ layers. Therefore, there is considerable interest in finding markers which are predictive of the pluripotent state.

A number of transcription factors that play a critical role in maintaining stem cell self-renewal have now been identified, and analysis of their expression is also used to characterize pluripotent cells. The POU transcription factor, OCT4, is expressed in pluripotent cell populations, such as early embryo cells, blastomeres, and germ cells. *In vitro,* OCT4 is expressed in pluripotent cells, such as EC, ES, and EGCs, and is down-regulated on differentiation. Furthermore, it has been demonstrated that OCT4 is necessary to retain pluripotency in murine embryonic cells, and that the level of expression can influence the status of pluripotency and direct the cells toward specific phenotypes. Several target genes for OCT4 have been identified, including UTF1, REX1, PDGFαR, OTX2, Lefty-1, and NANOG, but their exact roles in pluripotency and self-renewal are unclear. Other transcriptional regulators such as SOX2 act cooperatively with OCT4. The molecular mechanisms through which OCT4 and various co-factors act to regulate ESC pluripotency and self-renewal are complex, and are discussed in greater detail in a reviews by Niwa (2001) and Jaenisch and Young (2008).

Expression profiling has been utilized to identify functional markers associated with pluripotency. Initial

experimentation focused on the gold standard pluripotent cell, ESCs. Transcriptional profiles comparing mouse and human ESCs lines identified a set of 918 genes enriched in undifferentiated hESCs, compared to hESCs that had undergone differentiation. This set of genes included ligand–receptor pairs for the FGF, TGF-β–BMP, and Wnt signaling pathways. Some of these components corresponded to genes previously identified as "stemness" genes, indicating that a "core molecular program" may exist. In contrast, others have found a number of differences between expression profiles of mouse ESCs and human ESC lines using immunocytochemistry, reverse transcription-polymerase chain reaction (RT-PCR), and microarray analysis (Ginis *et al.*, 2004). As summarized in Table 38-1, ESCs from both species expressed transcription factors associated with pluripotent cells, such as OCT4, SOX2, TERT, UTF1, and REX1. Using RT-PCR and immunocytochemistry, significant differences were found in expression of vimentin, β-tubulin, α-fetoprotein, eomesodermin, HEB, ARNT, FOXD3, and the LIF receptor. Focused microarrays revealed profound differences in cell cycle regulation, control of apoptosis, and cytokine expression. These findings confirm that there are many differences between mouse and human ESCs, and demonstrate the need to use multiple methodologies to characterize these differences.

In order to identify a specific molecular signature associated with pluripotency, various groups have compared different pluripotent cell populations. Several groups have evaluated multiple hESC lines using microarrays, SAGE analysis, and transcriptional profiling to define "stemness". Rao and colleagues (Bhattacharya *et al.* 2004) proposed a set of pluripotency genes of around one hundred, based on transcriptional profiling. More recently, with the availability of increasing numbers of human pluripotent stem cell populations, researchers have begun defining regulatory networks and molecular signatures which are specific to pluripotent, multipotent, and unipotent stem cells. This has led to an unbiased metanalysis of multiple cell populations, and identified a set of genes associated with pluripotent cell populations termed PluriNet (Muller *et al.*, 2008). These data are intriguing, and demonstrate progress toward the identification pluripotent cell markers; however, broader application of this marker set is required to establish its predictive value.

Cytogenetic Analysis

Pluripotent cells have the capacity for prolonged cell proliferation *in vitro*, and the cytogenetic stability of these cells over time in culture is critical to their utility in both research and clinical applications. Currently, the most information about cytogenetic stability has been obtained from assessment of human ESCs. In most laboratories, this has been measured by G-banding, with most hESCs exhibiting a normal complement of chromosomes. There are now several reports in which maintenance of normal karyotype over long-term culture has been demonstrated. In contrast, some investigators have reported abnormal karyotypes in hESCs that are similar to those abnormalities observed in human ECs, such as trisomy 12 and 17. Further experimentation has suggested that, although hESCs maintain an apparently stable phenotype and karyotype over long-term culture, they are like other cells and can acquire mutations. Most recently, two separate groups have identified recurrent chromosomal abnormalities in hESCs maintained over long-term culture, including amplification at 20q11.21, which is associated with oncogenic transformation. It is important to note that abnormal cultures retain expression of standard hESC markers and pluripotency. It is unclear whether the presence of atypical cells in human ESC cultures will alter the fundamental characteristics of the cells or affect growth rate, cell cycle regulation, and capacity to differentiate; however, the occasional identification of these cells, albeit at a low frequency, highlights the importance of careful monitoring.

Differentiation

The fundamental characteristic of pluripotent cells is the ability to differentiate into cells of all three germ layers, which can be measured in several ways. Perhaps the most definitive test of pluripotency is the formation of chimeric mice. In this paradigm, ESCs are injected into the blastocyst, and the contribution of the ESCs to the resulting chimera is assessed to determine the differentiative capacity of the injected cells. Although this approach can assess mouse cells, this assay is clearly not suitable for human cells. Therefore, embryoid body (EB) formation *in vitro* and teratoma formation after injection into immunocompromised mice are currently used to measure pluripotency in the human system. This is accomplished by scoring the presence/absence of ectoderm, endoderm, and mesoderm in EBs and teratomas. However, quantitation of these assays has not yet been accomplished, making any quantitative evaluation of pluripotency in human cells particularly difficult. In order to develop more quantitative assays for differentiation of pluripotent cells, many groups are working on techniques to direct differentiation toward specific cell lineages. These "directed differentiation" techniques may ultimately allow for the assessment of the capacity to form specific cell types.

SUMMARY

Currently there are a number of human pluripotent cell populations that can be generated both from embryonic cell sources, and via reprogramming techniques. These different cell populations show similarities in expression of surface markers and transcription factors associated with pluripotency. To date, it remains unclear whether these markers will

be predictive of the differentiative capacity of the pluripotent cells. Current efforts are directed towards identifying a molecular signature which can be used prospectively to identify pluripotent cell populations. Continued characterization of the existing and newly-created pluripotent cell populations is required to understand the mechanisms involved in retaining the undifferentiated state of these cells, as well as the mechanisms involved in differentiation.

KEY WORDS

Aneuploidy Any deviation from the normal haploid number of chromosomes.

Karyotype A standard arrangement of the chromosome complement. A normal human female karyotype would have each of the 22 pairs of autosomes (non-sex chromosomes) arranged in numerical order together with the two X chromosomes.

Pluripotent The capacity to give rise to cells representing all germ layers.

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Chapter 39

Isolation and Maintenance of Murine Embryonic Stem Cells

Martin Evans

INTRODUCTION

Mouse embryonic stem (ES) cells were first isolated more than 20 years ago. Their isolation, and the conditions for their growth and maintenance, are well-described, both in the original references and in numerous reviews and methods texts. This chapter is, therefore, written more as a commentary than a cookbook.

I first discuss the growth and maintenance of cultures of mouse ES cells, because their isolation depends on the secure ability to maintain them. I would recommend that an established ES cell line be used to optimize growth conditions before attempts are made to isolate new ones.

ES cells grow well in culture, and are not particularly fastidious about the media, but it must be remembered that ES cell cultures are essentially primary cultures. Therefore, it is necessary to use conditions of tissue culture that maintain their primary properties, and do not select variants. Any growth in nonoptimal conditions will lead to selective pressure, and the appearance of better-growing but worse-differentiating strains. In some cases, these may be recognized as chromosomal variants, but in others there is no gross karyotypic change identifying the altered cell strain. They will certainly, however, be less able to differentiate and will produce abnormal chimeras.

MAINTENANCE OF EMBRYONIC STEM CELLS

There are major requirements for ES cell culture in the context of maintaining the totipotency necessary for genetic engineering and gene-targeting projects:

- The cells are maintained in their undifferentiated state.
- The cells maintain their normal capability and range of differentiation.
- The cells retain a normal karyotype, a prerequisite for germline transmission.

To maintain the stem cell state (suppression of differentiation), the cells need to be cultured with mitotically-inactivated feeder cells, or in the presence of leukemia inhibitory factor (LIF). Each method has advantages and disadvantages. Feeders provide a more robust approach, for the supplementation of media by growth factors will lead to a more pulsatile addition of the factors and will depend more on meticulous attention. Conversely, sufficient provision of feeders requires setting them up and inactivating at each passage. Growth in the presence of LIF may seem less troublesome, and makes certain types of experiments much easier, but it can have its disadvantages. Some ES cell lines seemingly grow better on feeders than in the presence of LIF, whereas others can be switched between the two conditions with little difficulty.

By far the best practical method for maintaining totipotent cells in tissue culture remains the use of fibroblast feeders (STO or primary embryo fibroblast). For any serious long-term investment in ES cells, it is wise to expand and freeze ES cells over several passages, checking the karyotype (and germline transmission) at each passage. Use should only be made of ES cells with proven karyotype and germline transmission properties for critical experiments.

ES cell cultures are essentially primary cultures, and it is necessary therefore to use conditions of tissue culture that maintain their primary properties, and do not select variants. It appears that extremes of growth conditions, either by plating the cells too sparsely or by allowing the cultures to become too dense and exhausting their medium, will encourage loss of the desired normal characteristics of ES cells. Either situation sets up conditions in which selection for abnormal (fast-growing, aneuploid) cells can occur. Healthy cultures of ES cells grow with a population doubling time of 15 to 20 hours. In practice, therefore, it is necessary to subculture ES cells about every three days, and to renew the medium regularly.

In as much as ES cells are not particularly fastidious about the medium in which they are grown, the optimum

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formulation I have used is Dulbecco's modified Eagles medium (DMEM) high-glucose, low-pyruvate formulation mixed 1:1 with Ham's F12M medium. This peculiar mix is a compromise between the high-yielding DMEM, originally designed for maximal growth of tissue-culture cell lines for virus production, and the finely balanced Ham's F12, originally developed for the clonal growth of cells. I originally supplemented the DMEM with nonessential amino acids (NEAA) and a mixture of nucleosides (adenosine, guanosine, cytidine, and uridine) to a final concentration of 30 mM, and thymidine to a final concentration of 10 mM. This supplement has not been tested properly when used in a DMEM/F12 mix, but it nevertheless may have an advantageous effect on the primary cell isolation. ES cells grow happily in DMEM alone, but they grow better in DMEM plus NEAA, and better still in DMEM/F12. The only disadvantage of the latter is that it becomes acidified faster, and the medium needs to be changed more frequently. These media may be prepared conveniently "in house" from premixed powder. Alternatively, 10 × concentrates may be used, or a medium may be bought ready to use at 1 × concentration. The water used must be of the highest purity, and glassware cleanliness is critical. These latter points have proved to be the most important in establishing new ES cell lines. I suspect that the most damaging contaminants are detergent residues, and that their effects are not necessarily seen in bulk passage cultures. The most useful tool for optimization of media, sera, and other conditions is the cloning efficiency test (explained later in this chapter), which should also be used for any troubleshooting.

MEDIA

The medium recommended for ES cells is as follows:

- DMEM/F12 (or DMEM)
- Glutamine (stored frozen as 100 × concentrate of 200 mM)
- b-mercaptoethanol to a final concentration of 10^{-4} M, either 4 ml neat in 500 ml or from a 10^{-2} M (100 ×) concentrate. (Prepared by adding 72 ml to 100 ml PBS)
- 10% calf serum and 10% foetal calf serum
- NEAA from 100 × concentrate
- Nucleosides from 100 × concentrate (if required)
- Antibiotics (if desired)—use only penicillin, streptomycin, kanamycin, or gentamicin. Do not use antimycotics.

SERA

Both fetal calf serum and newborn calf serum are used. It is a mistake to imagine that the higher price of fetal calf serum makes it better. Some newborn sera are excellent, and some fetal calf sera are extremely toxic to ES cells. Empirical selection is imperative. All serum should be carefully batch-tested using a cloning efficiency test (explained later in this chapter) and should be ordered in a sufficient lot for the experiments planned. Look both for lack of toxicity (aim for a cloning efficiency of 20% or more), and growth promotion (the size of colonies). Aim to buy new serum batches that equal or exceed the quality of the presently used (control) sera. Be cautious with sera that show toxicity at high (e.g., 40%) concentrations; they can be excellent at lower levels, but other sera can be just as good without extra toxicity.

COLONY-FORMING ASSAY FOR TESTING CULTURE CONDITIONS

The colony-forming assay described should be used to test all the components of the media, as well as some culture procedures (e.g., viability of cells after different regimens of dissociation):

- Set up experimental media in six-well cluster dishes (2 ml/well) at twice the intended final concentration of additives, and equilibrate in the incubator at 37ºC, 5% CO_2, in air before use. If you are using feeder cells, these need to be added either at this stage, or with the sample cells. Use duplicate wells for each condition and appropriate controls (e.g., a known "good" serum batch). For FCS batch testing, use growth media supplemented with the FCS batch at 5%, 10%, and 20%. All other components (e.g., mercaptoethanol concentrations and batches of media) may be tested in the same way.
- Disaggregate ES cell stock culture on day 2 after plating (i.e., semiconfluent), ensuring as close to a single-cell suspension as possible. In a test of sera, remember, if using trypsin, to inactivate by a wash in serum-containing medium, before redispensing into serum-free medium. Count and resuspend in growth medium at a density of 10^3 cells/ml.
- Add 2 ml cell suspension to each well (making 4 ml total volume 1 × concentration of additives), and return to the incubator.
- Incubate for six to eight days at 37ºC, 5% CO_2 in air.
- Fix, and then stain, plates with Giemsa. ES cell colonies can be identified by characteristic morphology and dark staining properties. Differentiated colonies are paler in color. (It is useful to check sample colony appearance before fixing and staining by inverted phase contrast.) Count the total number of colonies (the plating index), and the proportion of ES cell colonies. In tests of LIF or feeders, the maintenance of undifferentiated colonies is important.

I still recommend the use of inactivated feeder layers for the maintenance of ES cells. I have found that the attraction and convenience of feeder-free methods are outweighed by the frequency with which stocks deteriorate

under these conditions, and would therefore prefer that an important seed stock be maintained with feeders. It is possible to use a belt-and-braces policy—to use feeders and added LIF. For feeders, use either STO cells or primary mouse embryo fibroblasts.

STO cells are routinely grown in DMEM supplemented with 10% newborn calf serum. Remember that STO cells are effectively 3T3 cells, and should be passaged promptly when they reach confluence to prevent the accumulation of non-contact-inhibited cells in the population. Passage STOs at 5×10^3 cells per cm^2 and expect a harvest of up to 20 times this amount. If a confluent 10 cm Petri dish yields $>10^7$ cells, then the cells are losing their contact inhibition, and will no longer produce a good feeder layer. Either replace them with a new batch, or (and effectively) clone them out by seeding at ~100 cells per dish and select a flat clone to establish a new stock. Some workers prefer to use primary embryo fibroblasts to prepare feeder layers rather than STO cells, because they feel that better ES cell growth can be obtained. This may, however, reflect abused STO cells rather than an intrinsic superiority of primary embryo fibroblasts.

Feeders are prepared in the following manner:

- Remove the medium from a confluent 10 cm dish of STO cells, and replace it with DMEM/10% NCS plus 10 mg/ml of mitomycin C. (Stock mitomycin C is made at 2 mg/ml with PBS, and can be stored at 4ºC for two weeks.)
- Incubate plates for two to three hours. Avoid longer exposure to mitomycin C.
- Remove mitomycin C from the STO cells, and wash each plate three times with 10 ml PBS.
- Trypsinize, resuspend, and pellet the cells by centrifugation. This is an important washing step.
- Resuspend in growth medium, and seed onto gelatinized tissue culture plates at 5×10^4 cells per cm^2. These can be kept for later use (up to a week), or the inactivated cells can be stored for later use in suspension in a serum-containing medium at 4ºC for a similar time.

EMBRYONIC STEM CELL PASSAGE CULTURE

Passage and maintenance of the ES cells is straightforward; bear in mind the following. The cells should not be allowed to become so confluent that the medium becomes excessively acidified. Given the chance, they will continue dividing until they start to kill themselves by overcrowding and exhaustion of the medium; avoid this. Expect to harvest about 3×10^5 cells per square centimeter, and plate at $2 \times 10^4/cm^2$. Feed regularly and passage every three days. It is important that you seed the cells as a suspension of single cells with few, if any, aggregates. Such aggregates will initiate differentiation, and can severely complicate the ES culture for many purposes. Cells are best disaggregated by careful washing with PBS (remember, you have 20% serum in the medium, and this needs to be fully removed) followed by trypsin/EGTA (0.125% trypsin with 10^{-4} M EGTA in PBS—an improvement on trypsin/EDTA). Incubation at room temperature is sufficient. (It is also possible to disaggregate ES cells by a prolonged incubation in PBS/EGTA, but their viability is reduced.) Immediately after the trypsin incubation, add growth medium and pipette up and down a few times to generate single cells. Because it is important that the cells are well disaggregated, it is good to check by counting in a hemocytometer. You should see a mainly single-cell suspension with round, phase-bright cells. Dead cells or clumps are obvious; ragged and larger cells are feeders.

ISOLATION OF NEW EMBRYONIC STEM CELL LINES

Contrary to some well-expressed opinions, it is clearly possible to isolate ES cells from a variety of mouse strains, and not just from strain 129. I append a prescriptive description of ES cell isolation (given later in this chapter), but first it is useful to consider the background, and to point out that isolation is possible from the cleavage to early post-implantation stages and using different procedures.

Embryonic development starting from a single zygote proceeds through extensive cell proliferation and progressive cellular differentiation. At early stages some cells have wide prospective fates, but these are not necessarily self-renewing populations. Pluripotential stem cells were first identified experimentally in the mouse as the stem cell of teratocarcinomas by Kleinsmith and Pierce, who were able to passage the tumor by transferring single cells isolated from embryoid bodies. These testicular teratocarcinomas in mice arise spontaneously in specific genetic strains, and were extensively studied by Stevens (1970). The stem cells of these tumors arise from the primordial germ cells during gonad formation, and one question is whether this represents a parthenogenetic activation. This idea—that the teratocarcinoma stem cells might be embryo-related rather than germ cell-related—was tested directly by transplanting early embryos to adult testes or kidneys. Passageable, progressively growing teratocarcinomas were formed after transplantation of 1–3.5 day pre-implantation embryos. From some of these tumors, clonal *in vitro* differentiating embryonal carcinoma cell lines were isolated and characterized. These were the direct forerunners of ES cells and their isolation. They demonstrated their pluripotentiality by forming well-differentiated teratocarcinomas on reinjection into mice. These also differentiated extremely well *in vitro*, via an embryo-like route. A series of observations of the properties of these and similar embryonal carcinoma (EC) cells started to provide convincing evidence of their homology with the pluripotential cells in

the normal embryo, which were able to generate teratocarcinomas under conditions of ectopic transplantation. There seemed to be every reason to suppose that direct isolation into tissue culture should be possible.

Teratocarcinomas can also be formed from post-implantation mouse embryos. The embryonic part of the post-implantation mouse embryo undergoes gastrulation and forms an embryonic mesoderm by invagination from the ectoderm; this lies between the ectoderm and an embryonic endoderm. Skreb and his colleagues isolated these three layers of the rat embryo by microdissection, and tested their developmental potency by ectopic transplantation. Only the embryonic ectoderm produced teratomas with multiple tissue types and was hence pluripotential, but these were not progressively growing teratocarcinomas. When these experiments were repeated with gastrulating mouse embryos, it was discovered that, in contrast to the rat, transplantable teratocarcinomas were formed. At least, therefore, in the mouse, pluripotential stem cells could be recovered until about the end of gastrulation. Direct isolation of ES cells, however, from such a late stage has not been reported. Taking these comparisons and molecular data into consideration, we have argued that mouse ES cells are—regardless of the route of isolation—homologous to the early post-implantational epiblast, rather than the inner cell mass (ICM), as is often erroneously stated.

The culture conditions—feeder cells and media—had been refined by culturing both mouse and human teratocarcinoma EC cells. Evans and Kaufman, by using implantationally delayed blastocysts as the embryo source, succeeded in establishing cultures of pluripotential cells directly, without an *in vivo* tumor step. Subsequent studies showed isolation of such cell lines from both inbred and outbred strains, from normal 3.5-day blastocysts, and those that had been implantationally delayed. Martin, in an independent work, showed that isolated 3.5-day ICMs may also be used. These cells, which have become known as ES cells, share the properties of indefinite proliferative capacity, embryonic phenotype, and differentiative capacity with their forerunners—the EC cells; in addition, being primarily derived, they may be kept entirely karyotypically normal.

Eistetter reported that ES cell cultures may be established from disaggregated 16–21 cell morulae with an apparent immediate growth of the colonies from some of the explanted single cells. Up to four separate ES cell colonies were founded from a single embryo.

The latest stage of isolation into cultures of ES cells from embryos has been reported by Brook and Gardner, who used 4.5-day-old hatched, peri-implantational embryos flushed from the uterus. They microdissected the epiblast cells from both the primary endoderm and the trophectoderm. Both whole epiblasts and those that had been disaggregated into single cells readily generated ES cultures—if they were not left in contact with the endoderm. Brook and Gardner also reconfirmed the benefit of using delayed blastocysts, showing that the isolated epiblasts of these were the most efficient source of ES cell cultures.

METHOD FOR DERIVATION OF EMBRYONIC STEM CELLS

I would still recommend the original method of explantation of implantationally delayed blastocysts as the most effective method. It works well, and involves no microdissection or immunosurgery.

First, it is essential to pay attention to the optimization of the culture conditions, as described previously. If things don't work, suspect the purity of the media and particularly any possible contamination by detergent residues. Seemingly stupid problems, such as aliquoting the medium into small batches and using untested vessels (e.g., bijou bottles), can arise. Suspect specific items that have not been tested in the ES cell cultures—for instance, the cleanliness of the glass Pasteur pipettes from which micropipettes are pulled for manipulation of the embryos and growth of ES cell colonies.

Delayed blastocysts:

- Mate mice by caging together overnight and observing mating plugs in the morning. Separate the plugged females.
- On day 2 (counting the day of plug as day 0), it is necessary to remove estrogen activity but preserve progesterone. This was originally done by ovariectomy followed by injection of Depo-Provera, but we have introduced the less invasive, simpler, and considerably improved option of using the antiestrogenic effects of tamoxifen. The mice are treated with 10 mg tamoxifen and 1 mg Depo-Provera. Dissolve the tamoxifen in ethanol to make a 100 × stock, and dilute this in sesame oil before injecting a dose of 10 mg intraperitoneally. At the same time, administer a dose of 1 mg Depo-Provera subcutaneously. Kill the animals, and recover the delayed blastocysts by flushing from the uterus between day 6 and day 8.
- Prepare feeder layers preincubated in an ES cell medium. (Although it is routine to pretreat plastic Petri dishes with gelatine as an aid to ES cell culture, it is probably more important here than elsewhere.) I find that 1.6 cm four-well cluster dishes are convenient, but possibly better are 3 cm Petri dishes, each enclosed in a 10 cm plastic Petri dish (this aids handling and helps to minimize evaporation from the small volumes of medium).
- The embryos will enter diapause, and may be recovered by flushing from the uterus 6–12 days later. They will be large, slightly ragged, hatched blastocysts, usually with a visible ICM.
- Using a drawn-out Pasteur pipette, recover the blastocysts and place them on preincubated feeder layers in full ES cell medium.

- Incubate for about four days, observing daily.
- When the blastocyst has attached and spread out, with a visibly growing ICM derivative (but before this becomes encapsulated in a thick endodermal layer—if you see this happening, you have left it too long), carefully remove the medium and carefully wash twice in PBS with 10^{-4} M EGTA.
- Wash with PBS/EGTA containing 0.125% trypsin and aspirate, leaving a thin wetting layer of the trypsin solution. Very briefly incubate at 37ºC.
- Have a drawn-out Pasteur pipette with a tip aperture of 20–50 microns ready and filled with full ES cell medium. Observe under a dissecting microscope. When the cells are loosening, but before everything swims off the dish, carefully flood the medium over the ICM area from the drawn-out pipette and then suck it up.
- This should neatly disaggregate it into single cells and small (two- to four-cell) clumps—don't aim to make a complete single-cell suspension at this stage. Blow these cells out beneath the surface of the medium of a preincubated feeder dish or well in the ES cell medium.
- Incubate this dish for 7–10 days, observing carefully. Do not be tempted to feed daily, but if it seems necessary, replace about half the medium after five days. If many small ES cell colonies are becoming established, let them grow to a reasonable size before passaging 1:1 onto new feeders; thereafter, you should be able to grow it in the normal manner. (The size of the colonies depends on the size of the cell aggregate that founded them—if they have come from a single cell, they can be left for 10 days before passage.) If few ES cell colonies appear, it is useful to repeat the trypsinization and passage by drawn-out Pasteur pipette on each one, before trusting a bulk passage.

With 129 mice and similar strains, and if all is going well, you should be able to establish new ES cell lines from up to half of the explanted blastocysts. It is useful to make a freeze stock at the earliest possibility. There are usually plentiful cells by passage 4–6.

SUMMARY

Although it is relatively easy to create new cell lines, their full validation—stability in culture, karyotype, chimera-forming ability, and germline potential—takes a lot of time and work. It is, therefore, important to have sufficient characterized stocks of particular passage and to freeze batches to allow useful repeatable studies to be undertaken.

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Chapter 40

Isolation, Characterization and Maintenance of Primate Embryonic Stem Cells

Michal Amit and Joseph Itskovitz-Eldor

INTRODUCTION

During the 1970s, multipotent cell lines were isolated from the stem cells of mouse teratocarcinomas. Some of these embryonal carcinoma (EC) cell lines were shown to differentiate *in vitro* into a variety of cell types, including muscle and nerve cells. When grown in suspension, EC cells tend to aggregate into cell clusters known as embryoid bodies (EBs), in which part of the cells differentiate spontaneously. They had also been shown to differentiate *in vivo*, forming teratocarcinomas following their injection into recipient mice. EC cells served for years as models for research on development, establishing all the methodological know-how needed for the isolation and maintenance of embryonic stem (ES) cells (reviewed by Andrews, 2001).

The first mammalian ES cell lines were derived by Evans and Kaufman in 1981 from the mouse blastocysts. Since then, mouse ES (mES) cells have played a key role in developmental studies. Unlike EC cells, ES cells are regarded as pluripotent, thus possessing the ability to differentiate into each cell type of the adult body. Following their injection into blastocysts, mES cells were reported to integrate into all fetal germ layers, including the germline; in some cases, they were reported to develop into mature chimeric animals. Furthermore, several mES cell lines had been shown to form entire viable newborns, when injected into tetraploid embryos or heat-treated blastocysts (reviewed by Smith, 2001).

Because of the unique features of mES cells and their proven differentiative abilities, much effort has been invested in the derivation of ES cell lines from additional animal species. ES cell lines and ES cell-like lines have been successfully isolated from rodents, such as golden hamsters, rats, rabbits, and also domestic animals three nonhuman primates (Amit and Itskovitz-Eldor, 2003). None of the ES cell lines cited here have been shown to demonstrate all features of mES cells; the bovine ES cell lines were not shown to create teratomas following injection into severe combined immunodeficiency (SCID) mice. The ability of nonhuman primate ES cell lines to integrate into all three germ layers during embryonic development using the blastocyst injection model has not been tested. Therefore, the mES cell model is still the most potent research model among all existing ES cell lines.

The first successful derivation of human ES cell (hES) lines was reported by Thomson and Marshall in 1998. The availability of hES cell lines provides a unique new research tool, with widespread potential clinical applications (Figure 40-1). Human ES cells may be used for various study areas, such as early human development, differentiation processes, and lineage commitment. The availability of differentiation models of hES cells to specific lineages, similar to the existing ones for mES cells, may lead to future uses of these cells in cell-based therapy, and for industrial purposes.

Since the first report on hES cell isolation to date, additional groups have reported on the derivation of hES cell lines, and up until now there are at least 500 published cell lines (Strulovici *et al.*, 2007). The increasing numbers of available hES cell lines indicate that the derivation of these lines is a reproducible procedure with reasonable success rates.

Lately, a new source for pluripotent cells was developed when Yamanaka and colleagues demonstrated the ability to reprogram mouse, and later on human, somatic cells, to ESC-like cells (Takahasi and Yamanaka, 2006; Takahasi *et al.*, 2007). The somatic cells were reprogrammed into pluripotent ones by overexpression of four transcription factors: c-Myc; Oct4; Flf4; and Sox2 by induced infection, These reprogrammed cells, also termed induced pluripotent stem (iPS) cells, have been demonstrated to exhibit many features of hES cells, including expression of typical

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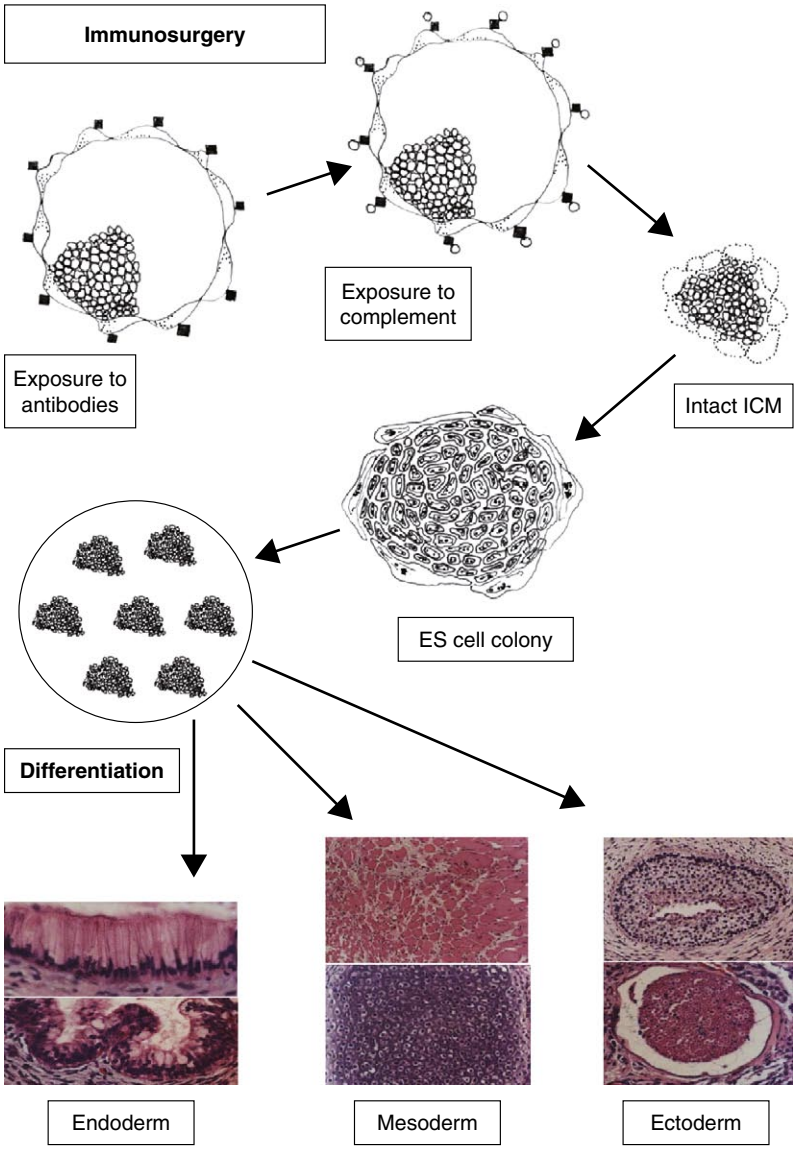


FIGURE 40-1 Derivation, differentiation and possible applications of hES cells. For the isolation of hES cell lines, the ICM of the blastocyst is selectively removed and further cultured on mitotically-inactivated MEF. When removed from the MEFs feeder layer, the resulting hES cells may differentiate into representative tissues of the three embryonic germ layers both *in vitro* and *in vivo*.

ES cell markers, similar colony and single-cell morphology, the ability to differentiate into representative tissues of the three embryonic germ layers both *in vitro* and *in vivo*, and chimera formation following blastocyst injection. Since the initial formation of iPS cells, the methodologies of their derivation have been significantly improved, either by removing the originally-used oncogenes (c-Myc), or by using transfection, rather than infection methods. Several somatic cells were used for iPS cell derivation, including embryonic or fetal fibroblasts, foreskin fibroblasts, adult dermal and skin tissue, and adult liver and stomach cells. Future studies will reveal which of the existing culture and differentiation protocols developed for hESCs are also suitable for these cells. This chapter focuses on techniques for the derivation, characterization, maintenance, and differentiation of primate ES cells.

WHAT ARE PRIMATE EMBRYONIC STEM CELLS?

Based on the well-characterized mES cells, a list of ES cells features was created, which includes the following criteria. ES cells:

1. can be isolated from the inner cell mass (ICM) of the blastocyst;
2. are capable of prolonged undifferentiated proliferation in culture;
3. exhibit and maintain normal diploid karyotypes;
4. are pluripotent, i.e., are able to differentiate into derivatives of the three embryonic germ layers;
5. are able to integrate into all fetal tissues during embryonic development following injection into the blastocyst, including the germ layer;

TABLE 40-1 Characteristics of Primate Embryonic Stem Cells

1. Isolated from the ICM of the blastocyst
2. Capable of prolonged undifferentiated proliferation in culture.
3. Exhibit and maintain normal diploid karyotype.
4. Pluripotent, i.e., able to differentiate into derivatives of the three embryonic germ layers, even after being cultured continuously at the undifferentiated stage.
5. Clonogenic, i.e., each single ES cell has the ability to form a homogeneous line harboring all parental ES cell line features.
6. Express high levels of *OCT 4*—a transcription factor known to be involved in the self-maintenance process of ES cells.

6. are clonogenic, i.e., single ES cells have the ability to form a homogeneous line harboring all parental ES cell line features;
7. express high levels of *OCT 4*—a transcription factor known to be involved in their process of self-maintenance;
8. can be induced to differentiate after continuous culture at the undifferentiated state;
9. remain in the S-phase of the cell cycle for most of their lifespan; and
10. do not show X-chromosome inactivation (Smith, 2001; Hall *et al.*, 2008) (illustrated in Table 40-1).

According to accumulating knowledge, primate ES cells meet most of these criteria. Both human and nonhuman primate ES cell lines were derived from embryos at the blastocyst stage (summarized in Table 40-2). All primate ES cell lines were shown to be capable of continuous culture at an undifferentiated state, but expression of high levels of *OCT 4* had been demonstrated only for hES cells. The morphology of primate ES cells resembles that of mES cells: they create small and round colonies, although primate ES cell colonies seem somewhat less dense (Figure 40-2). On the single-cell level, there is no notable difference: like mES cells, primate ES cells are small and round, exhibiting a high nucleus-to-cytoplasm ratio, with one or more prominent nucleoli and typical spacing between the cells (Figure 40-2). Their pluripotency had been demonstrated both *in vitro*, by the formation of Ebs, and *in vivo* in teratomas in which the cells differentiated into all three primary germ-layer derivatives. The karyotypes of primate ES cell lines were found to be normal diploid karyotypes, even after prolonged culture. Cases of karyotype instability are scarce, suggesting that they represent random changes, which often occur in cell culture. Primate ES cells have also been shown to be clonogenic, with resultant single-cell clones demonstrating all ES cell features (Amit and Itskovitz, 2003).

For ethical reasons, the ability of hES cells to integrate into fetal tissues during embryonic development cannot be tested. Mouse ES cells remain in the S-phase of the cell cycle for the majority of their lifespan. Up until now, the specific stage of the cell cycle in which primate ES cells spend most of their lifespan has not been reported. In addition, it has been demonstrated that, similarly to mES cells, hES cells do not exhibit X-inactivation. When maintained in the undifferentiated stage, both X chromosomes are active and, on differentiation, one chromosome undergoes inactivation. Recently, some of the existing hES cells lines were found to vary in their X-inactivation status, and undifferentiated hES cells were shown to exhibit X-inactivation. This may be indicative of a different source for some of the lines, other than the ICM.

The potential of primate ES cells in the research on early primate embryonic development, and the development of research models for chronic diseases and for transplantation medicine, encourage scientists to invest great efforts in the isolation of nonhuman primate ES cells. The list and features of the reported nonhuman primate ES cell lines are summarized in Table 40-2. To date, there are more than 100 established and well-characterized hES cell lines in several laboratories worldwide.

TECHNIQUES FOR THE DERIVATION OF HUMAN EMBRYONIC STEM CELL LINES

The derivation of hES cell lines is a relatively simple procedure, with success rates of up to 30% and 50% for mice and humans, respectively. Embryonic cell lines are derived from embryos at the blastocyst stage. The isolation can be conducted using three methods: immunosurgical isolation; mechanical isolation; or the use of an intact embryo.

Immunosurgical Isolation

In the 1970s, Solter and Knowles developed a procedure known as immunosurgery for the selective isolation of ICM cells from the blastocyst needed for research on early embryonic development. This method of selectively isolating the ICM from the blastocyst laid the groundwork for the first ES cell line derivation in 1981 from mouse blastocysts.

The process of immunosurgery includes several stages. Initially, the glycoprotein outer layer of the zona pellucida (ZP) is dissolved by Tyrodes solution or pronase enzyme. The exposed embryo is then incubated for approximately 30 minutes in antihuman whole serum antibodies, which attach to any human cell. Penetration of the antibodies into the blastocyst is prevented because of cell–cell connections within the outer layer of the trophoblasts, leaving the ICM untouched. After rinsing off any antibody residue the blastocyst is transferred into a guinea pig complement-containing medium, and incubated once more until cell lysis is notable (see the schematic drawing of the procedure in Figure 40-1, and pictures in Figure 40-3A–C).

TABLE 40-2 Characteristics of the Existing Nonhuman Primate Embryonic Stem Cell Lines

Line	Karyotype	EBs formation	Formation of teratomas	Continuous culture	Staining with undifferentiating markers	Reference
R278 Rhesus ES cells	42, XY	Not available	+	>12 months	+	Thomson *et al.*, 1995 and 1998
R366 Rhesus ES cells	42, XY	+	+	>3 months	+	Thomson *et al.*, 1995 and 1998
R367 Rhesus ES cells	42, XY	Not available	+	>3 months	+	Thomson *et al.*, 1995 and 1998
R394 Rhesus ES cells	42, XX	Not available	+	Not available	Not available	Thomson *et al.*, 1998
R420 Rhesus ES cells	42, XX	Not available	+	Not available	Not available	Thomson *et al.*, 1998
R456 Rhesus ES cells	42, XX	Not available	Not available	Not available	Not available	Thomson *et al.*, 1998
R460 Rhesus ES cells	42, XY	Not available	Not available	Not available	Not available	Thomson *et al.* 1998
Cj11 Marmoset ES cells	46, XX	+	Not available	>12 months	+	Thomson *et al.* 1996
Cj25 Marmoset ES cells	46, XX	+	Not available	>3 months	Not available	Thomson *et al.* 1996
Cj28 Marmoset ES cells	46, XY	+	Not available	>3 months	Not available	Thomson *et al.* 1996
Cj33 Marmoset ES cells	46, XX	+	Not available	>3 months	Not available	Thomson *et al.* 1996
Cj35 Marmoset ES cells	46, XX	+	Not available	>3 months	Not available	Thomson *et al.* 1996
Cj36 Marmoset ES cells	46, XX	+	Not available	>3 months	Not available	Thomson *et al.* 1996
Cj39 Marmoset ES cells	46, XX	+	Not available	>3 months	Not available	Thomson *et al.* 1996
Cj62 Marmoset ES cells	46, XX	+	Not available	>12 months	+	Thomson *et al.* 1996
CMK5 Cynomolgus monkey	40, XY	+	+	>3 months	+	Suemori *et al.*, 2001
CMK6 Cynomolgus monkey	40, XY	+	+	>6 months	+	Suemori *et al.*, 2001
CMK7 Cynomolgus monkey	40, XX	+	Not available	>month	+	Suemori *et al.*, 2001
CMK9 Cynomolgus monkey	40, XX	+	Not available	>3months	+	Suemori *et al.*, 2001

The following references were used:

Thomson *et al., Proc. Nat. Acad. Sci. USA, 92,* ***7844–7848, 1995.***

Thomson *et al., Biol. Reprod., 55,* ***254–259, 1996 [checked original].***

Thomson and Marshall, *Curr. Top. Dev. Biol., 38,* ***133–165, 1998 [checked original].***

Suemori *et al., Dev. Dyn., 222,* ***273–279, 2001.***

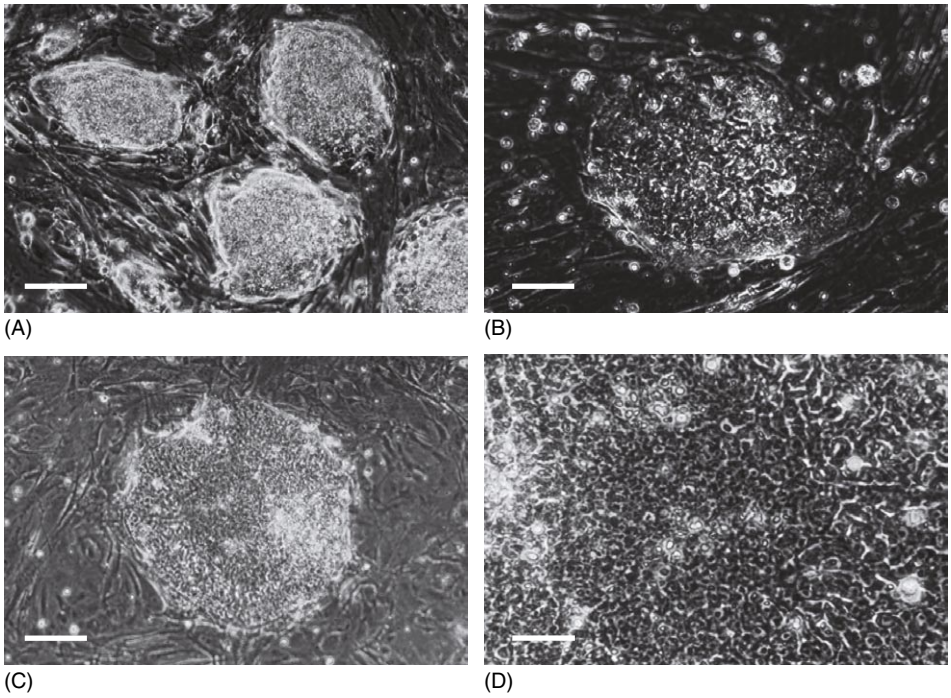


FIGURE 40-2 Colonies of undifferentiated cells from different ES cell lines grown on MEFs. (A) Mouse ES cell colony; (B) Rhesus ES cell colony; and (C) Human ES cell colony. (D) High magnification photograph of hES cells; note the high nucleus-to-cytoplasm ratio, the presence of one or more nucleoli, and the typical spacing between the cells.

Since the ZP allows the penetration of both antibodies and guinea pig complement, it may be alternatively removed postlysis by complement proteins. Following the selective removal of the trophectoderm, the intact ICM is further cultured on mitotically-inactivated mouse embryonic fibroblasts (Figure 40-3).

Mechanical Isolation

ES cell lines can be derived directly from cultured blastocysts, either by the mechanical dissection and partial removal of the trophoblast layer with 27G needles (Figure 40-3D) or pulled Pasteur pipettes, or by plating a zona-free whole embryo on mitotically-inactivated MEFs. When trophoblasts are only partially or are not removed, embryos attach to the feeder layer and flatten, permitting continuous growth of the ICM, with the remaining surrounding trophoblasts as a monolayer. When the ICM reaches a sufficient size, it is selectively removed and propagated (Figure 40-3E). This method, however, yields significantly lower success rates compared to immunosurgery, due to the ICM cells' tendency to differentiate.

"Late Stage" Embryos

Early mammalian embryonic development *in vivo* requires the division of the blastocyst's ICM into two layers shortly after implantation: a layer of primitive endoderm, which generates the extraembryonic endoderm; and a layer of primitive ectoderm, which generates the embryo and some extraembryonic derivatives. The mammalian embryo continues to develop; at day 14 post-fertilization, gastrulation occurs in which cells become progressively restricted to a specific lineage. Thus the pluripotency of these cells is lost. In other words, pluripotent or totipotent cells exist and proliferate in the mammalian embryo for a short window of time, whose exact borders were never defined.

Mouse ES-like cell lines were recently isolated from embryos at the epiblast stage (Tesar *et al.*, 2007; Brons *et al.*, 2007). The isolated epiblast cells (EpiSC) grow as an epithelial colony and proliferate, resembling the morphology of hES cell colonies. The resulting cells express most of the known ES cell features, including expression of transcription factors associated with pluripotency, such as Oct3/4 and Nanog. They also efficiently form EBs while cultured in suspension, and are able to create teratomas when injected into athymic nude mice. Nevertheless, some differences were found between these late stage cells and ES cells. EpiSCs were found to express genes FG F5 and Nodal, which are specific to the late epiblast layer post-implantation. Gene expression analysis demonstrated that EpiSC are distinct from mES cells, and from mES cells in their epigenetic state and in the signals controlling their differentiation.

The pluripotency of human post-implantation embryonic cells, between the time of implantation and the gastrulation process, has never been examined previously. Following their plating in whole on MEFs, as mentioned earlier, embryos most often flatten, and a small ICM colony, surrounded by a monolayer of trophoblasts, can be observed (Figure 40-3E). In some cases, however, the ICM

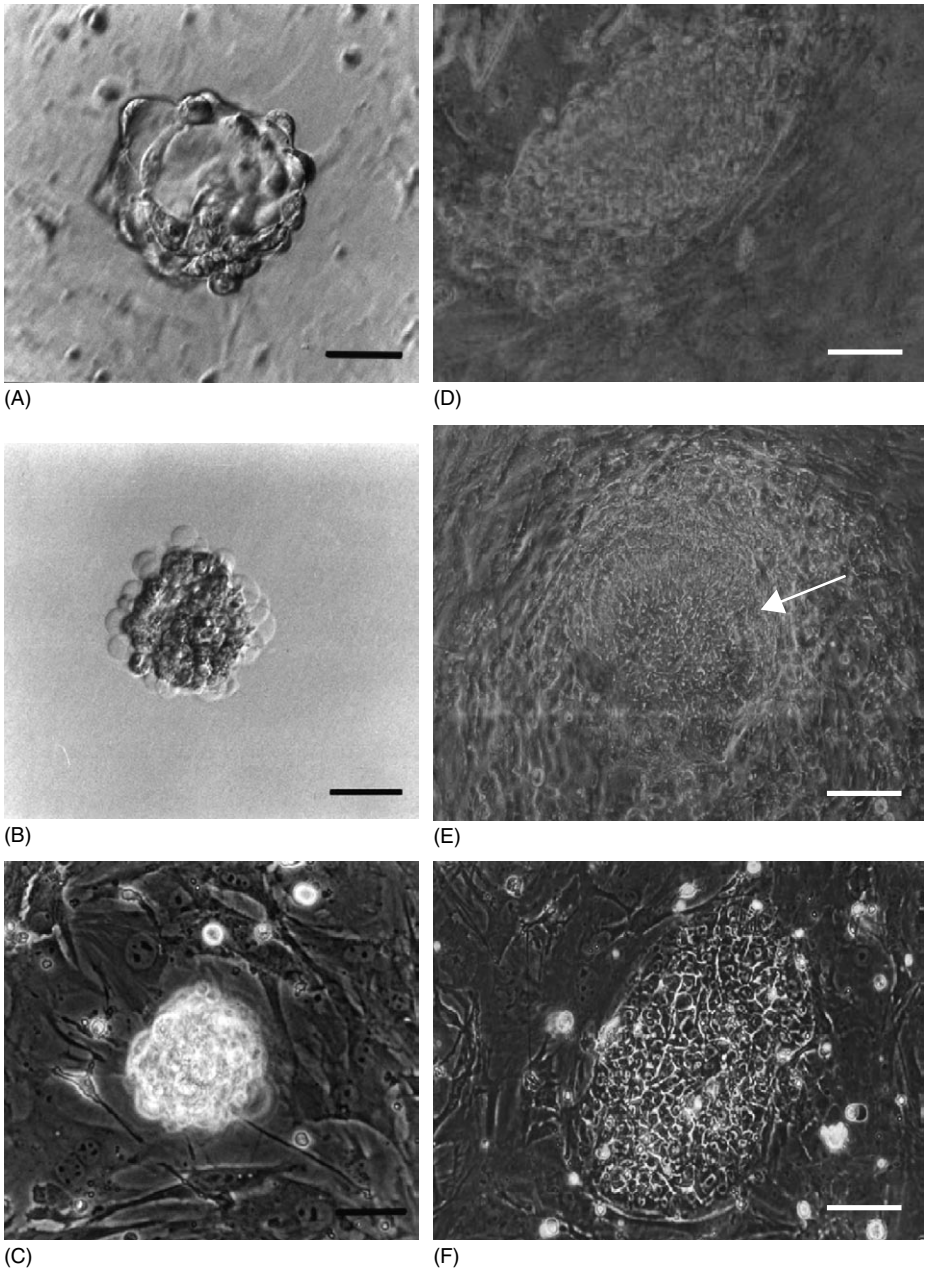


FIGURE 40-3 Methods for hES cell lines derivation. (A) Human blastocyst after ZP removal by Tyrode's solution, during exposure to rabbit antihuman whole antiserum; (B) Embryo after exposure to guinea pig complement; the ICM is surrounded by cells after lysis; (C) The intact ICM on mitotically inactivated MEFs; (D) Growing ICM after mechanical removal of the trophoblast; (E) Embryo placed in whole on MEFs after six days of culture. The growing ICM (white arrow) is surrounded by fibroblast-like cells; (F) Resulting hES cells colony cultured on MEFs. Bar = 50μm. Pictures A–C are from Amit & Itskovitz, (2002). *J. Anat.*, *200*, 225–232.

continues to grow and to develop small cysts. Small and flat structures of pluripotent-resembling cells can be recognized. Once selectively removed, these cells are further cultured and propagated on fresh MEF plates. The resulting cell line exhibited the main characteristics of hES cell lines: (1) prolonged undifferentiated proliferation; (2) pluripotency, i.e., the creation of EBs when grown in suspension or teratomas after injection into SCID beige mice; (3) maintenance of normal karyotypes after being continuously cultured for several months; and (4) partial or full expression of typical surface markers. The key question arising, of whether pluripotent cells derived from "post-implantation" embryos are significantly different from hES cells isolated using the traditional methods has no clear answer so far. Further research is needed to fully characterize these cell lines.

Derivation of Human Embryonic Stem Cell Lines Harboring Specific Genetic Defects

The ability of hES cells to differentiate into each cell type of the adult body may be used for research on the nature and course of specific diseases. Models established by the use of hES cell lines carrying specific genetic defects may

be highly effective in the development of drug or gene therapy designed to treat these diseases.

Two methods of obtaining such lines are genetic manipulation of existing hES cell lines or derivation of hES cell lines from genetically compromised embryos. Since the chance that donated surplus embryos from the *in vitro* fertilization (IVF) program carry genetic diseases is relatively low, the preferable source of donated embryos would be nonretrieved embryos from the preimplantation genetic diagnosis (PGD) program. PGD is designed for couples that are carriers of genetic diseases to ensure the transfer of healthy embryos to the uterus by their examination prior to implantation. For this purpose, the embryos are grown *in vitro* to the six to eight-cell stage, at which point one or two cells are removed and analyzed, either by polymerase chain reaction (PCR) or by fluorescence *in situ* hybridization (FISH).

In our experience, post-PGD embryos continue to develop *in vitro* to the blastocyst stage. Five hES cell lines that were isolated from donated post-PGD blastocysts were derived and found to possess hES cell features. One line was found to harbor the Van Waardenburg disease (deletion at the PAX3 gene), and the other had myotonic dystrophy. The cell lines carrying genetic diseases could therefore be used for the development of *in vitro* models for these disorders.

It has already been shown that iPS cells can be derived from adult fibroblasts isolated from biopsies of patients carrying specific diseases. Thus, the availability of iPS cells presents a new source for genetically abnormal cell lines, whose derivation can be used to develop models for studying the diseases' mechanisms and for drug testing and discovery (Park *et al*, 2008).

DERIVATION OF HUMAN EMBRYONIC STEM CELL SUBCLONES

Human embryonic stem cell lines are derived from the ICM, which may not represent a homogenous cell population. To eliminate the possibility that the pluripotency of the lines reflects a collection of several distinct committed multipotential cell types in the culture, parental lines have to be single-cell cloned. The main aim of the first derivation of hES single-cell clones was to establish the parental lines' pluripotency, but as with mES cells, single-cell cloning has additional advantages.

Method for the Derivation of Single-cell Clones

To derive single-cell clones, hES cells are trypsinized to single cells, and each cell is plated in a separate well of 96-well plates. Alternatively, single cells may be plated in separate culture cylinders. After approximately two weeks of growth, the resulting colonies are passaged and propagated. When cloning is conducted on hES cells, the rates of success highly depend on the culture medium. Several culture media were tested to clone the first parental hES cell lines: medium supplemented with either FBS or serum replacement, and either with or without human recombinant basic fibroblast growth factor (bFGF). The highest cloning rates were obtained when serum-free growth conditions supplemented with bFGF were used. Although success rates were relatively low (up to 1%), they have been found to be the most suitable conditions for the clonal derivation of hES single-cell lines. Human ES single-cell lines, H-9.1, and H-9.2 derived by Amit *et al.* in 2000 under these conditions maintained ES cells features: they proliferated continuously as undifferentiated cells for prolonged periods (eight months); maintained stable and normal karyotypes; differentiated into advanced derivatives of all three embryonic germ layers *in vitro* in EBs and *in vivo* in teratomas; and expressed high levels of telomerase activity. Thus the pluripotency of hES single cells has been established.

To date, many single-cell clones from five parental ES cell lines, H-1, H-9, H-13, I-3, and I-6, have been derived. Interestingly, in the same culture conditions, most parental lines had the same cloning efficiency of 0.5%, with two exceptions: line H-1 had a lower cloning efficiency of 0.16%, and the cloning of line I-4 proved unsuccessful. The variation among the existing parental cell lines in respect to their cloning efficiency remains to be determined.

Recently, several agents were found to increase the cloning efficiency of hES cells by folds. One such agent is ROCK inhibitor, which is used prior to trypsinization (Watanabe *et al.*, 2007). The increased success rate of single-cell cloning enables the routine use of this method.

Advantages of Single-cell Clones

In addition to proving the pluripotency of single hES cells, single-cell clones may have further features. First, they are easier to grow and manipulate than the parental lines. Second, they form homogeneous cell populations, which may be instrumental for the development of research models based on gene knockout or targeted recombination. The transfected or knockout cells could be cloned and analyzed individually. Clones that express the desired genotype could be further cultured and used for research. The main disadvantage of this strategy is the relatively low cloning efficiency (0.5–1%), which results in a rather difficult model to obtain when coupled with reduced successful recombination rates. The advantage of this strategy, though, lies in the prolonged culture abilities, which make extended periods of research use possible once a satisfactorily manipulated single-cell clone is created.

Any future application of hES cells for scientific or therapeutic purposes will depend on their karyotypic stability. The first reports on hES cell line derivation specifically state that, like mES cells, their karyotypes remain normal after continuous culture. Although in some culture conditions hES cell lines retain normal karyotypes even after prolonged culture of over 107 passages, random karyotypic instability may still occur. The karyotype of one culture of line I-6, for instance, became abnormal after 150 passages (30 months) of continuous culture on MEFs. Since the first reports on hES cell line derivations, reports on karyotypic instability have accumulated. Amit *et al.* examined the karyotype of parental line H-9 after 7, 8, 10, and 13 months of continuous culture, and found that only once after seven months of continuous culture, four out of the 20 cells examined demonstrated abnormal karyotypes. Eiges and colleagues reported in 2001 on two cells with 17-chromosome trisomy in a stably transfected clone. Based on the growing data on hES cells, and the experience with mES cells, it is reasonable to assume that a subpopulation with an abnormal karyotype will acquire a selective growth advantage and take over the culture. Therefore, the periodical cloning of cultured hES cells for the purpose of maintaining a homogenous euploid population may be needed, but will be infrequent.

METHODS FOR HUMAN EMBRYONIC STEM CELL CULTURE

The traditional methods used for culturing hES cells have not changed dramatically since their development for the derivation and culture of ECs and mES cells (for more information, read *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* by E. J. Robertson (1987)). Unlike mES cells which can be cultured on gelatin with the addition of leukemia inhibitory factor (LIF) to the culture medium, until recently, hES cells could be cultured only on mitotically inactivated MEFs. The feeder layer has a dual role: first, as the term implies, the MEFs support ES cell growth; second, the MEFs prevent spontaneous differentiation of ES cells during culture. Although hES cells require meticulous care, they can be cultured in large numbers, and frozen and thawed with reasonable survival rates (for more information see *Human Embryonic Stem Cells*, A. Y. Chiu and M. Rao (Eds)). Since their first derivation in 1998, the culture systems of hES cells have enjoyed three major advantages in the basic culture conditions: (1) Amit *et al.* demonstrated the ability to grow these cells under serum-free conditions in 2000; (2) Richards and colleague reported the use of human feeder layers as substitutes to MEFs in 2003; and (3) the maintenance of the cells in an undifferentiated state in feeder-free conditions was reported in the study of Xu *et al.* in 2001.

Human Feeders

In the future, hES cells may be directly applied in cell-based therapies. Any clinical use of these cells will require compliance with FDA guidelines. During hES cell culture on MEFs with medium supplemented with FBS, there is a risk of exposing the cells to retroviruses or other pathogens. One of the solutions to this problem is the isolation and culture of hES cells in an entirely animal-free environment.

Recently, Richards and colleagues presented an animal-free system for the production and growth of hES cell lines. In the culture medium proposed, FBS was replaced with a supplement of 20% human serum and a human feeder layer replaced the MEFs. Co-culture with both human embryo-derived feeder layers or Fallopian tube epithelial feeder layers was found to support hES cell growth and isolation of an hES cell line.

Another animal-free culture system for hES cells reported by Amit *et al.* consisting of foreskin feeder layers and a medium supplemented with serum replacement. After more than 105 passages (more than 300 doublings), the three hES cell lines grown in these conditions, I-3, I-6, and H-9, exhibited all hES cells features, including expression of typical surface markers and transcription factor *Oct 4*, differentiation into representative tissues of the three embryonic germ layers both in EBs and in teratomas, high telomerase activity after 46 passages of culture, and maintenance of normal karyotypes. The morphology of the hES cell colony grown on foreskin feeder layer is slightly different than that grown on MEFs, in terms of its long and elliptic organization (Figure 40-4A). No difference was found among the 12 foreskin fibroblast lines tested for their ability to support prolonged and undifferentiated proliferation of hES cells. A recent publication by Hovatta and colleague demonstrated the derivation of new hES cell lines using foreskin fibroblasts as feeders and a medium supplemented with fetal calf serum.

There are several advantages to the use of foreskin fibroblasts as feeder layers. The serum replacement enables better-defined culture conditions. Furthermore, unlike embryo-derived or Fallopian tube epithelial human feeder layers which can grow to reach a certain limited passage, foreskin fibroblasts can grow to 42 passages, or alternatively can be immortalized using traditional methods and still support hES cell culture. While exploring different foreskin fibroblasts lines, no difference was found between the ability of high-passage human foreskin feeders or immortalized ones and the ability of low-passage ones to support the growth of hES cells, even after several cycles of freezing and thawing. These feeders may therefore have an advantage when pathogens screening is essential. Although these culture systems reflect a step forward towards animal-free conditions, they cannot be regarded as well-defined, because the need for culturing hES cells on

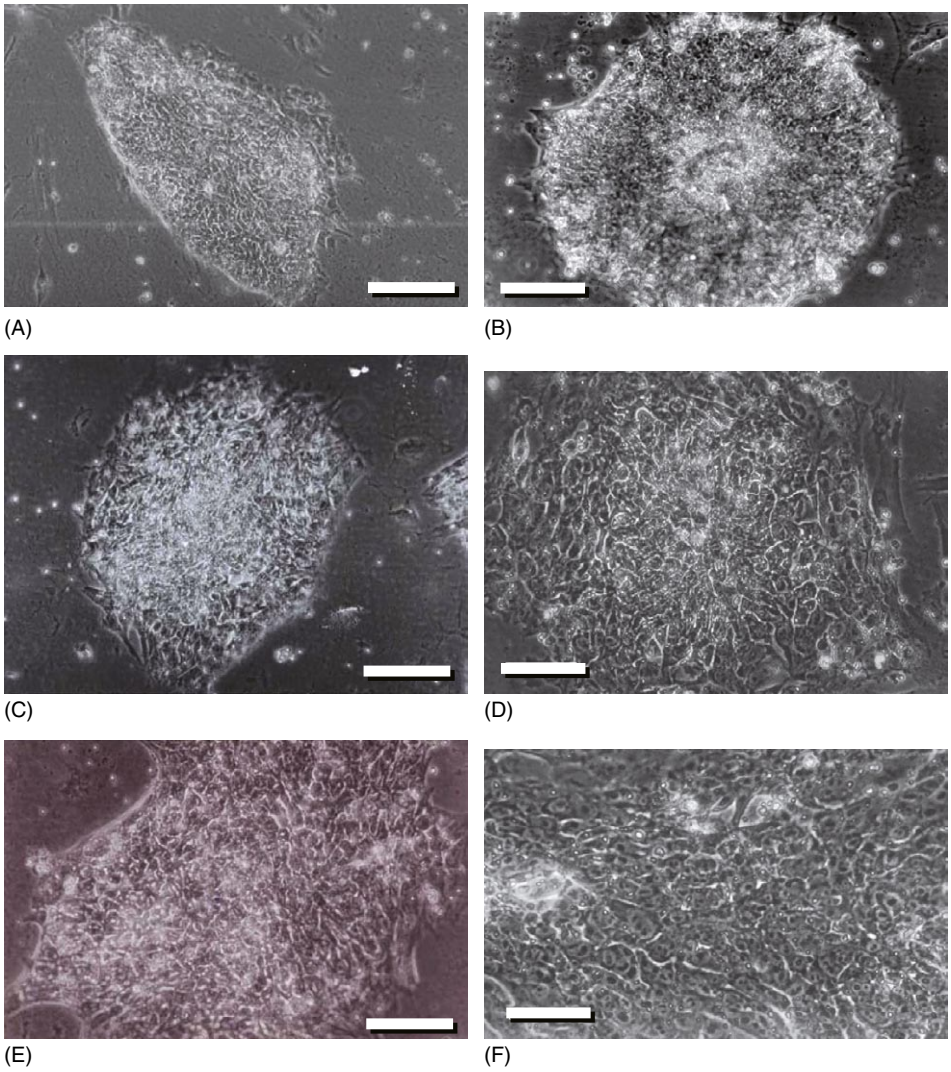


FIGURE 40-4 Human ES cell colonies grown in different culture conditions. (A) Human ES cell colony from line I-6 after several passages on foreskin matrix. Bar = 75 μM; (B) Example of the morphology of an undifferentiated ES cell colony from clone I-3.2, grown for 12 passages on Matrigel matrix using MEF conditioned medium. Bar = 50 μM; (C) Human ES cell colony from line I-6 after several passages on MEF matrix. Bar = 75 μM; (D) Colony I-3 grown in feeder free conditions for 21 passages. Bar = 50 μM; (E) Human ES cell colony from line I-6 after several passages on Matrigel. Bar = 75 μM. No condition medium added; (F) Cells from cell line I-3 grown in feeder-free conditions for 20 passages. Bar = 38 μM.

feeder cells will limit the large-scale culture of hESCs, and increase batch-to-batch variability.

Feeder-free Culture of Human Embryonic Stem Cells

For large-scale growth and therapeutic applications, the ideal culture method for hES cells would be to grow them on an animal-free matrix using a serum-free medium. The first step toward this solution was reported by Xu *et al.*, who demonstrated a culture system in which hES cells were grown on Matrigel, laminin, or fibronectin using 100% MEF-conditioned medium, supplemented with serum replacement. The major shortcomings of this system are the possible exposure of the cells to mouse pathogens through the condition medium, the absence of a well-defined condition medium, and the need for a simultaneous growth of MEFs. Richards *et al.* also tested the ability of human feeder-conditioned medium (embryo-derived, Fallopian tube epithelial, or foreskin fibroblast feeder layers) to sustain a continuous undifferentiated proliferation of hES cells grown on collagen I, human extracellular matrix, or laminin. These conditions were found inferior to the use of human feeder cells.

During recent years, extensive work has been conducted to further improve the feeder-layer free culture system of hES cells. Several agents were reported to support undifferentiated hES cell cultures in feeder layer free conditions, including the combined TGFβ1 and bFGF, Activin, high levels of Noggin or bFGF, and a blend of five factors used in defined culture media (summarized by Amit and Itskovitz-Eldor, 2009). The variability of these systems implies that more than one pathway is involved in maintaining hES cells in the undifferentiated state. Further study is required, in order to clarify the mechanism underlying the activity of these factors in hES cell self-maintenance. Most of the suggested feeder-layer free culture

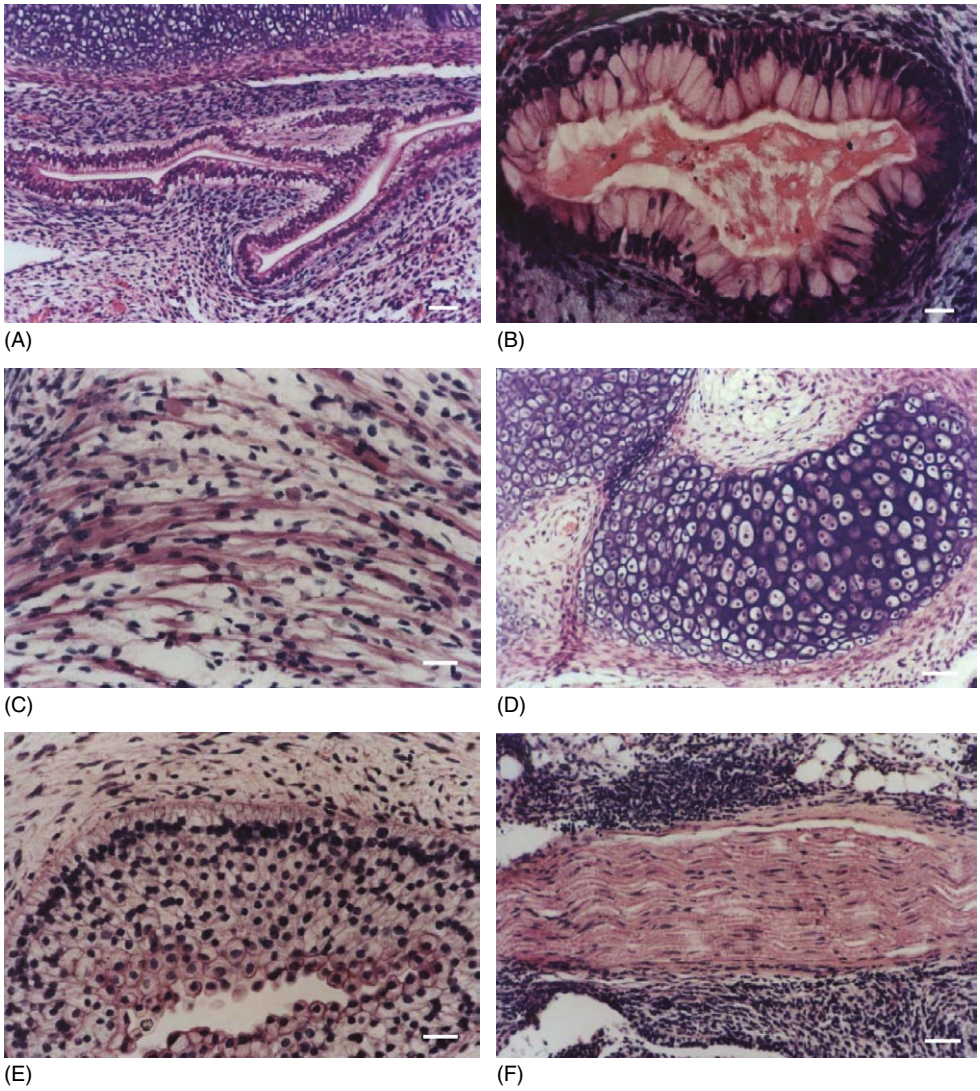


FIGURE 40-5 Histological sections from teratomas formed by hESCs grown on Matrigel, MEF matrix, or fibronectin in SCID-beige mice. (A) Columnar epithelium; (B) Mucus-secreting surface epithelium resembling the stratified epithelium found in stomach; (C) Embryonal myotubes; (D) Cartilage; (E) Stratified epithelium; (F) Myelinated nerve. Hematoxylin and eosin staining. For A, D, E and F Bar = 50μM, for B and C Bar = 25μM.

systems were not reported to be used with nonhuman primate ES cell culture, and the available publications on these cell cultures still focus on the use of feeder layers.

Within the reported hES cell lines, only two publications demonstrated a supportive layer-free culture system to derived new hES cell lines. The first report by Klimanskaya and colleagues described an MEF-produced matrix and medium supplemented with a high concentration of bFGF, LIF, serum replacement, and plasmanate (Klimanskaya *et al.*, 2005). Six new hES cell lines were successfully derived exhibiting ESC features after prolonged culture of more than 30 passages. Although the culture system included non-defined and animal materials, those pioneering works prove it is feasible to derive hES cell lines without feeder layers. A more recent publication by Ludwig and colleagues reported the derivation of two new hES cell lines using a defined serum-free and animal-free medium, in feeder layer-free culture conditions (Ludwig *et al.*, 2006). The matrix consisted of human collagen, fibronectin, and laminin, and the medium was supplemented with a combination of five growth factors. Although the newly-derived cell lines sustained most hES cell features, the two new hES cell lines were reported to harbor karyotype abnormalities. It is yet to be determined whether these abnormalities were the result of exceptional events of karyotypic irregularities that sometimes occur during prolonged cultures, or whether that culture system simply cannot support karyotype stability.

PRIMATE VERSUS MOUSE EMBRYONIC STEM CELLS

Although primate and mES cells share most of the features of ES cells, there are some differences between them. The most distinct difference is the colonies' morphology. Unlike mES cells, which form compact and piled colonies, all primate ES cells were reported to form a "flat"

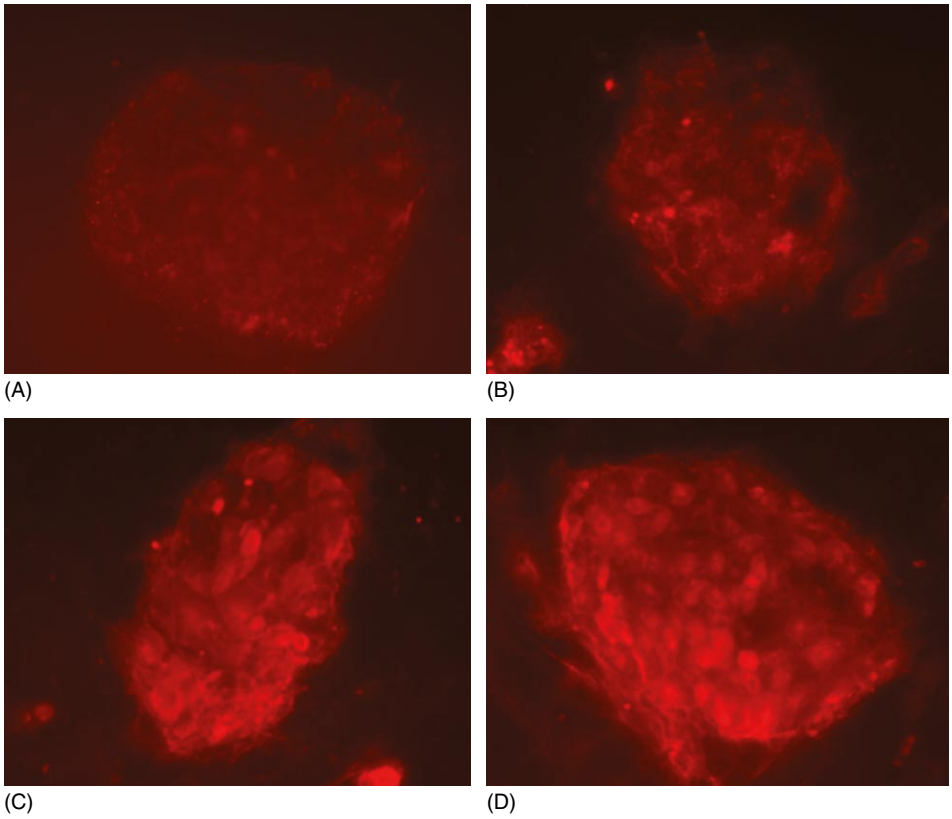


FIGURE 40-6 Immunostaining of ES cells with typical surface markers. (A) Mouse ES cell colony stained with SSEA-1; (B) Rhesus ES cells stained with SSEA4; and (C) TRA-1-60; (D). I-8 hES cell colony stained with TRA-81.

morphology (Figure 40-2). On the single-cell level, however, there is no difference: both mouse and primate ES cells demonstrate typical spaces between the cells, a high nucleus-to-cytoplasm ratio, and the presence of one or more nucleoli (Figure 40-2).

Several surface markers were shown to be related to the undifferentiated stage of cells. Primate ES cells have been found to express different surface markers than mES cells. Although mES cells highly express surface marker stage-specific embryonic antigen-1 (SSEA-1), nonhuman primate ES cells and hES cells do not express this marker. In addition, hES cells and nonhuman primate ES cells strongly express SSEA-4, tumor recognition antigen (TRA)-1-60, and TRA-1-81; and they weakly express SSEA-3, with the exception of cynomolgus ES cells, which do not express SSEA-3, mES cells never express these markers. Examples of the surface antigen expression are demonstrated in Figure 40-6.

It had been reported by Rastan and Robertson in 1985 that most mES cell lines are 40, XY. The reduced numbers of XX mES cell lines were related in this study to the lack of X-chromosome inactivation and X-chromosome deletions in mES cells. Interestingly, no difference was found in the XX-to-XY ratio among primate ES cells lines. This finding may be linked to different timing of X-chromosome inactivation during the early embryonic development of the mouse and primate.

LIF was found by Williams *et al.* to maintain undifferentiated proliferation of mES cells with the absence of a MEF feeder layer. Both hES cells and nonhuman primate ES cell lines failed to maintain undifferentiated morphology without an MEF feeder layer and with LIF. This finding may indicate that different pathways govern the self-maintenance procedures of ES cells isolated from different species.

Another remarkable difference between mES cells and primate ES cells is the latter's ability to differentiate into trophoblasts *in vitro*, a characteristic that rarely appears in mES cells. Mouse trophoblasts do not secrete chorionic gonadotropin (CG), a hormone that was shown to be a crucial signal for maternal recognition of pregnancy secreted by the trophoectoderm. Both rhesus and marmoset ES cells were shown by Thomson and colleague to secrete increasing levels of CG when allowed to differentiate. The cynomolgus ES cells do not secrete CG, as been demonstrated by Suemori and co-workers. Human ES cells were reported by Thomson and colleague to differentiate into hCG secreting trophoblasts *in vitro*.

The differences between mouse and primate ES cells may reflect the differences in the embryonic development of these species. Therefore, mES cells may be found less suitable for research on early primate embryonic development. One of the advantages of using nonhuman primate ES cells is their potential contribution to the creation

of transgenic monkeys, which may serve as models for chronic illnesses. (For additional reading on the differences between mouse and human ES cells, read Thomson and Marshall, 1998.)

PRIMATE EMBRYONIC STEM CELL DIFFERENTIATION SYSTEMS

During their two decades of existence, many *in vitro* differentiation models were developed for mES cells. Similar models are gradually being created for primate ES cells. Most existing methods for directing mES cells differentiation include the formation of EBs as one of the initial steps, as is the case with the differentiation of mES cells into hematopoietic cells, neurons, and cardiomyocytes. Apparently this step encourages ES cells to differentiate, and consequently increases the rate and efficiency of differentiation. Itskovitz-Eldor and colleague reported that human ES cells, like mES cells, spontaneously create EBs when cultured in suspension, including cystic EBs, which contain derivatives of the three embryonic germ layers. All nonhuman primate ES cell lines were also reported to create EBs *in vitro*, although some of them seemed less organized than mouse-derived EBs.

Primate ES cells can also differentiate *in vivo*. Following their injection into the hind muscle of SCID mice, they spontaneously create teratomas, in which they differentiate into representative tissues of the three embryonic germ layers. They have been shown to differentiate into bone and cartilage tissue, striated muscles, gut-like structures, structures resembling fetal glomeruli, neural rosettes, etc. In Ebs, ES cells differentiate as groups of cells or into simple structures, in teratomas, primate ES cells can create more complex and well-organized organ-like structures, such as hair follicles, salivary glands, and teeth buds. The development of organ-like structures in teratomas requires cooperation between cells and tissues derived from different germ layers. (For additional information on mES cell differentiation, see Smith review from 2000; and on human ES cells see chapters 8–12 in *Human Embryonic Stem Cells*, Chiu and Rao (Eds)).

One of the milestones in creating directed-differentiation systems for mES cells was to genetically manipulate these cells. The efficient and stable transfection models can be used both for creating pure populations of cells, and for inducing directed differentiation. Additional uses may be to "flag" the cells to permit their recognition in histological sections, and for research models aimed at examining the role of specific genes during specific differentiation or embryonic development.

Stable transfection protocols were already developed for hES cells. In a report from Eiges and colleague (2001), various transfection agents were used, while Ma *et al.* and Gropp and co-workers used lentiviruses vectors. Similar models were developed by the research groups of Asano and Hanazono for nonhuman primate ES cells.

To date, several studies have been published on lineage-specific differentiation of nonhuman primate ES cells in both spontaneous and directed-differentiation models. These include induced differentiation into hematopoietic cells using bone morphogenic protein 4, spontaneous differentiation into endoderm derivatives, and spontaneous and induced differentiation into neural cells.

Human ES cells had been shown to spontaneously differentiate into cardiomyocytes, endothelial cells, and insulin-secreting cells. Directed differentiation models for hES cells include neural precursors, hematopoietic cells, cardiomyocytes, trophoblasts, and hepatocytes. The data presented here demonstrate the feasibility of developing differentiation models for primate ES cells similar to those of mES cells.

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Chapter 41

Approaches for Derivation and Maintenance of Human Embryonic Stem Cells: Detailed Procedures and Alternatives

Irina Klimanskaya and Jill McMahon

INTRODUCTION

Although derivation of mouse ES cell lines has become a common procedure, the limited number and quality of the currently available hES cell lines is testament to the difficulties encountered at various stages of their derivation and maintenance. The current techniques used for the maintenance of hES cells require scrupulous attention to the details of cell culture systems including: quality control of the reagents; monitoring of growth conditions; and an ongoing assessment of the pluripotent status of the cells in order to promote consistency in the cell culture system. Distributors of the available hES cell lines frequently recommend attending special training courses before working with the hES cell lines they provide.

The authors have experience in derivation and culture of over 30 hES cell lines, including hES cells derived without feeder cells, and lines established from single blastomeres (Cowan *et al.*, 2004; Klimanskaya *et al.*, 2005, 2006, 2007; Chung *et al.*, 2008). All our lines have been adapted to trypsinization, which makes passaging of the cells less labor-intensive and generates cells in sufficient numbers to permit experimentation. All of these lines can be successfully frozen and thawed, using very simple procedures, with a recovery rate of 10–30% or higher.

Techniques for deriving and maintaining pluripotent ES cells in culture have been described by a variety of laboratories, and there are similarities and vital differences. For the derivation and maintenance of hES cell lines in our laboratory we adapted previously published methods, and developed an approach which has consistently produced new cell lines and has been successfully taught to many researchers around the world.

The goal of this chapter is to describe aspects of derivation and maintenance of hES cells that we found to be critical for generating hES cell lines. We respond to questions received from other researchers with regard to: equipment, preparation, and quality control of media and reagents; cell passaging techniques; and other aspects of hES cell morphology and behavior.

SETTING UP THE LABORATORY

Equipment

Initial steps in the derivation process are conducted under a dissecting microscope. We make an effort to keep the embryos and the dishes containing the early mechanically passaged dispersions at 37ºC. Dishes brought out of the incubators are set on 37ºC slide warmers, or viewed using microscopes fitted with heated stages. The mechanical dispersions necessitate having the dishes open for extended periods, and since the cultures are vulnerable to contamination at this time, we have the dissecting microscope within a bench top laminar flow hood. A high quality stereo microscope with a wide range zoom is essential for the mechanical dispersion of colonies, including the ICM outgrowth; it permits an overall assessment of each plate, and evaluation of the morphology of each colony when doing the mechanical passaging.

The equipment that is used in our laboratory is as follows:

- Stereomicroscope for microdissection: Nikon SMZ-1500 with the magnification range of 10–100×. This works well, with its easy zoom and the positioning mirror that

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regulates the depth and contrast of the image. A whole 35 mm dish can be scanned for colony morphology, and the zoom permits selection of the parts of the colony that are the best for dissection with precision.
- Inverted microscope: A Nikon TE 300, or any similar microscope, set up with phase contrast and objectives 4×, 10×, 20×, 40×, and Hoffman 20×, 40×. Recommended for fine observation of hES cells, and HMC is required for embryo evaluation.
- Heated microscope stage for both stereo and inverted microscope: (Nikon).
- Slide warmer keeps plates at 37ºC during observation and mechanical dispersions.
- Bench-top laminar flow hood with a HEPA filter. Vertical hoods by Terra Universal (Anaheim, Calfornia) are a good inexpensive choice. Mid-Atlantic Diagnostics (Mount Laurel, New Jersey) manufactures workstations that include a biosafety cabinet and a built-in dissecting microscope; these models are more expensive but meet the GMP requirements, if necessary. We found these vertical hoods were tall enough to accommodate a dissecting microscope, and were very convenient and reliable. Horizontal models sometimes produce too much vibration.
- Reliable tissue culture incubator. All parameters (CO_2 concentration, humidity, and temperature) need to be checked daily with external monitoring equipment.

External monitoring equipment:

- Surface thermometer;
- Mercury liquid-immersed thermometer;
- Hygrometer;
- CO_2 monitor and gas calibration kit (GD 444 SR-B by CEA Instruments, Emerson, New Jersey).

Quality Assurance of Equipment

Consistency in growth conditions is very important for the development of the embryos and growth of hES cells. The checklist of parameters which are monitored daily includes the percentage of CO_2 (5.0%), temperature (37ºC), and humidity of the incubators (>90%). A checklist with daily readings is very helpful for timely recalibration if any undesirable trend is noticed. Warming stages are constantly monitored with surface thermometers.

The incubators are checked prior to any new derivation round by growing mouse embryos from the two-cell stage to blastocyst; a passing score requires 50% to go to blastocyst. Ideally, until the newly-derived lines can be frozen, the cultures should be split between two incubators, to prevent the loss of a line in the case of equipment failure. If hES cells need to be derived and maintained under the conditions of good manufacturing practices (GMP), GMP protocols for equipment and reagent validation would have to be followed.

Sterility

There are certain aspects of hES cell line derivation which put the associated work under more stringent sterility requirements than any typical cell culture laboratory. Among these is the nature of human embryos, each being unique and very valuable. Because of the labor-intensive nature of derivation and expansion, the new lines usually require a team effort until they are safely frozen. Experiments may require long periods in culture, such as in the course of cell differentiation into embryoid bodies, which often takes several weeks. In addition, because hES cells are prone to spontaneous differentiation under unfavorable conditions, many laboratories prefer not to use antibiotics in cell culture media.

The reagents should either come sterile from the manufacturer, or be filter-sterilized in the laboratory. Most of the cell culture supplies can be bought sterile. However, everything that is in-house sterilized by autoclaving, gas, or dry heat needs to be quality-controlled. We use biological indicators (spore strips from Steris, Mentor, Ohio). If it comes to the worst, a triple-action drug Normocin (active against gram-negative and gram-positive bacteria, fungi, and mycoplasma) appears to be well-tolerated by hES cells, without significant changes in their pluripotency or growth rate, and often permits the rescue of contaminated cultures.

PREPARING AND SCREENING REAGENTS

Media Components

- KO-DMEM (Invitrogen cat #10829).
- DMEM high glucose (Invitrogen cat #11960-044).
- Knockout Serum Replacement™ (KSR, Invitrogen cat #10828). Each lot needs to be tested; but, as a guide, we found the lots with osmolarity higher than 470 mOsm/kg and endotoxicity lower than 0.9 EU/ml were the best. Upon thawing, make single-use aliquots and freeze.
- Plasmanate (Talecric, Research Triangle Park, North Carolina). Each lot needs to be tested.
- FBS (Hyclone cat # SH30070.02). Each lot needs to be tested. Heat-inactivate at 55ºC for 30 minutes, if desired, and freeze in aliquots.
- Beta-mercaptoethanol, 1000 × solution (Invitrogen cat # 21985-023).
- Non-essential amino acids (NEAA), 100 × solution (Invitrogen cat #11140050).
- Penicillin/streptomycin, 100 × solution (Invitrogen cat #15070-063).
- Glutamax-I, 100 × solution, a stable dipeptide of L-glutamine and L-alanyl, a glutamine substitute (Invitrogen cat #35050-061).

- Penicillin/streptomycin and Glutamax-I are kept in frozen single-use aliquots.
- bFGF (Invitrogen cat #13256-029). Add 1.25 ml of protein-containing medium (we use basal medium, supplemented with Plasmanate and Serum Replacement, as described below) to a vial containing 10 μg of bFGF. This makes 8 μg/ml of stock solution. Increasing final bFGF concentration to 8–20 ng/ml can be beneficial for the cells, especially at early stages of derivation, after thawing or when the cells are grown at low density. Make 120 μl aliquots and freeze.
- Human LIF (Millipore International cat # LIF1010).
- 0.05% trypsin/0.53 mM EDTA (Invitrogen cat #25300-054).
- Gelatin from porcine skin (Sigma, cat. # G1880).
- PBS, Ca^{2+}, Mg^{2+}-free (Invitrogen cat #14190-144).
- Normocin, antibiotic active against Gram +/− bacteria, also has anti-mycoplasma and anti-fungi activity (Invivogen, San Diego, California; cat # ant-nr-2; comes as a 500 × solution).

Media Recipes

Bottles of media that are opened frequently become alkaline rapidly, we suggest making smaller quantities that will last approximately a week.

Primary Mouse Embryo Fibroblast Growth Medium

To a 500 ml bottle of high glucose DMEM add:

- 6 ml penicillin/streptomycin;
- 6 ml Glutamax-1;
- 50 ml FBS.

Human Embryonic Stem Cell Basal Medium (BM)

To a 500 ml bottle of KO-DMEM add:

- 6 ml penicillin/streptomycin;
- 6 ml Glutamax-1;
- 6 ml NEAA;
- 0.6 ml beta-mercaptoethanol. (NOTE: This volume pertains to the 1000 × diluted solution (see the list of reagents), not to 100% beta-mercaptoethanol).

Human Embryonic Stem Cell Derivation Medium

Use this medium at early stages of ICM outgrowth. It has higher LIF and bFGF concentration, and contains fetal bovine serum (FBS). You can switch to hESC growth medium when a steady growth of colonies has been reached (usually, passage 2–4).

To 100 ml of basal medium (bM) add:

- 5 ml Plasmanate;
- 5 ml KSR;
- 5 ml FBS;
- 240 μl of human LIF (final concentration 20 ng/ml);
- 120 μl of bFGF stock solution (final concentration 8 ng/ml) or more (up to 20 ng/ml);
- Sterilize by 0.22 μm filtration.

Alternatively, two media can be prepared: one containing Serum Replacement and Plasmanate only, the other one containing only FBS (10–15%). Mix them 1:1 or 2:1 for the earliest stages of derivation. FBS may help to stimulate the hES cell colony growth, but may reduce its content if any undesirable spontaneous differentiation is observed.

We also supplement derivation medium with hES-cell-conditioned medium. To produce it, derivation medium without FBS is added to an almost confluent culture of hES cells of very good morphology (see below) incubated overnight, collected, filtered through 0.22um filters and used at 25–30%.

Human Embryonic Stem Cell Growth Medium (hESCM)

To 200 ml of basal medium add:

- 20 ml Plasmanate;
- 20 ml KSR;
- 240 μl of human LIF for 10 ng/ml or 480 μl for 20 ng/ml;
- 120 μl of bFGF stock solution (final concentration 4 ng/ml) for 1 × bFGF or more, if a higher concentration is desired;
- Sterilize by 0.22 um filtration.

Gelatin:

Dissolve 0.5 g of gelatin in 500 ml of warm (50–60ºC) Milli-Q water. Cool down to room temperature, sterilize by 0.22 μm filtration; makes 0.1% solution. Treat dishes by flooding plates and leaving solution at room temperature for 30 minutes, aspirate solution prior to use.

Mitomycin C:

Add 2 ml of sterile Milli-Q water to a vial (2 mg) of lyophilized Mitomycin C (Sigma, cat. # M 0503); makes 1 mg/ml stock solution. The solution is light-sensitive and is good for one week at 4ºC.

Screening Media Components

It is important to be consistent with the screening, aliquoting, and storage of the components of the media. Various lots of KSR, Plasmanate, and FBS should be screened, preferably on hES cells. The screening of Serum Replacement lots should be done prior to the first batch running out, so

that an evaluation of its qualities can be compared side-by-side to the previous lot. Some newly-derived hES cells will completely die out with a change in lots at the initial stages.

Screening of Fetal Bovine Serum, Plasmanate, or Knockout Serum Replacement

This test is based on a published procedure for screening FBS lots for mouse ES cell work (Robertson, 1987, p. 74). This approach can be used for screening any combination of reagents or finding the best concentrations for media supplements. The quality of the reagents is assessed by counting the number of colonies, evaluating the morphology of the hES cells, and by staining for alkaline phosphatase activity as detected with a Vector Red Kit (Vector Laboratories, Burlingame, California).

1. Prepare 12-well plates with PMEF. For each lot tested, you will need at least 12 wells to vary the concentration of the component being tested, from the working concentration to high enough concentrations to evaluate toxicity: 8%, 10%, 20%, and 30%. Each concentration is done in triplicate and compared to known media components at working concentrations.

2. Split hES cells onto 12-well plates with a ratio of 1:6–1:10. The difference in reagents will be more noticeable when the cells are started at low density, however, some cell lines grow very slowly and differentiate when they are kept at a low density, therefore adjust the splitting ratio to the specific hES cell line. Resuspend the cells in a small volume of basal medium, and add equal volumes of cell suspension to each well of the test plate; pipette up and down in each well or slowly move the plate in perpendicular directions for even distribution of the cells. Do not rotate, as doing so will move most of the freshly added cells to center.

3. Change the medium daily, and evaluate colony morphology under the microscope. Human ES cells grow in flat, tightly-packed colonies with sharp refractory borders. The colonies appear deep red when stained for alkaline phosphatase activity. In differentiating colonies, the cells are more loosely packed with diffuse borders, and are pinker in comparison. Usually, the difference between the conditions being tested becomes more obvious as colonies grow bigger; however, as the colonies grow larger they can begin to touch each other, and they have a tendency to differentiate. Staining one of the triplicate wells for alkaline phosphatase activity prior to seeing signs of differentiation is advisable. Continue with the other wells in each set for another day or two before staining (see Figure 41-1 for a sample test). This test can also be used to compare performance of different media formulations or new products offered commercially; 8% KSR/8% Plasmanate medium can be used as a control. Adequate record-keeping

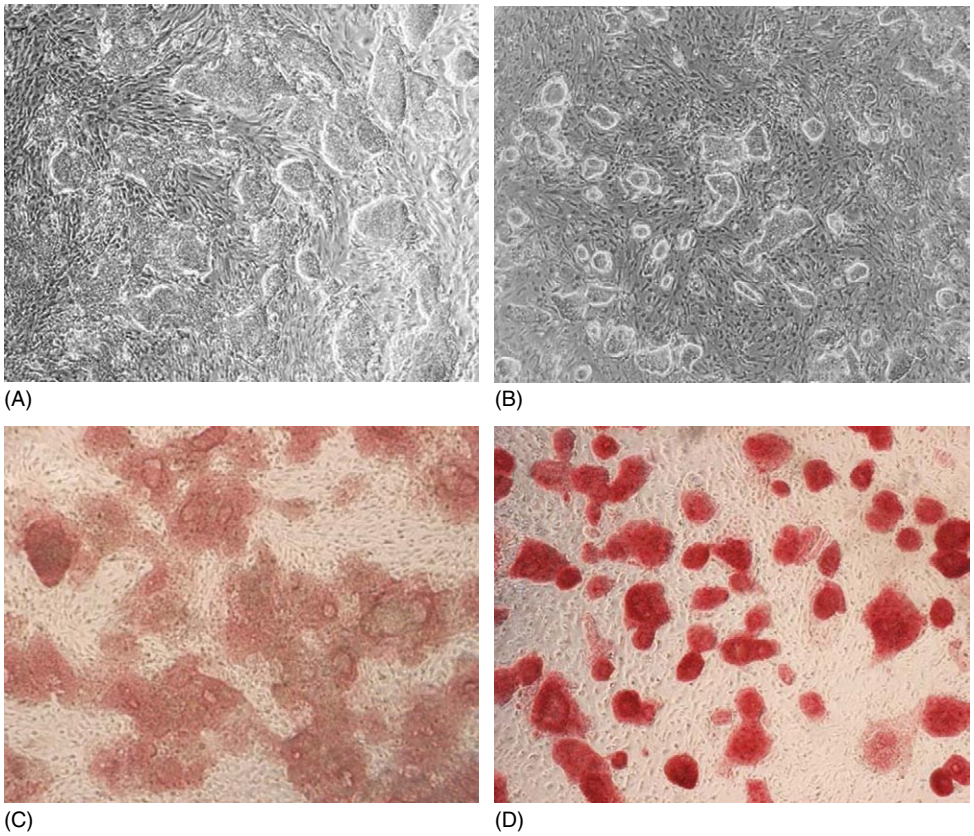


FIGURE 41-1 Media testing. Comparison of 16% Serum Replacement (A, C), with 8% Serum Replacement; 8% Plasmanate (B, D). Quality of the media supplements is assessed by the morphology of the colonies in bright field (A, B), as described in the text, followed by staining for alkaline phosphatase activity (C, D). Note that although the morphology of the colonies in (A) and (B) are comparable, the activity of alkaline phosphatase is higher in medium with both Serum Replacement and Plasmanate (D). Magnification × 40.

for all commercial and in-house prepared lots of reagents is essential for troubleshooting, should the performance of hES cells begin to fail.

PREPARING PRIMARY MOUSE EMBRYO FIBROBLAST (PMEF) FEEDERS

We grow all of our hES cells on PMEF feeders that have been Mitomycin C treated to generate stable monolayers. The PMEF are made by standard procedures using 12.5 dpc ICR mouse embryos (Robertson, 1987, p. 77). The 12.5 dpc embryos are eviscerated, but the heads are left on during tissue disruption in trypsin; plating density is 1.5 embryos per 150 mm plate. PMEF are expanded once after the initial plating (1:5 split), and then frozen (P1). The growth rate of PMEF and their performance as feeders decreases as they go through multiple passages; therefore, thawed PMEF are only passaged once (P2) for expansion purposes prior to Mitomycin C treatment, at which point a new vial of PMEF would be thawed.

Mitomycin C Treatment and Plating

Mitomycin C is added to the medium of a confluent plate of PMEF at a concentration of 10 μg/ml and incubated at 37ºC for three hours. The cells are harvested by trypsinization, and plated in PMEF medium onto gelatin-coated plates. In serum-free hESC growth media, the PMEF may appear less confluent due to the spindle-like form the cells adopt; to ensure a confluent monolayer we recommend a plating density of 50–60 000 cells/cm^2. We replace the PMEF medium with hESC growth medium the day after plating, and use plates of PMEF no longer than three to four days after Mitomycin C treatment. We have noticed that some lots of Mitomycin C may be inconsistent—the reagent forms an insoluble precipitate, and the characteristic purple color of the solution lightens dramatically. Reduced concentration of Mitomycin C causes proliferation of "treated" PMEF which results in their detachment in a few days and/or formation of gaps in the monolayer where the drops of medium hit the plate. Correctly inactivated PMEF do not form such gaps, and can remain intact on the plate for up to three to four weeks. Such old PMEF are not good for hES cell culture, are but useful for preparation of extracellular matrix plates for feeder-free culture (Klimanskaya *et al.*, 2005).

MECHANICAL PASSAGING OF HUMAN EMBRYONIC STEM CELL COLONIES

Many established hES cell lines are passaged with collagenase, or by dispase in conjunction with mechanical dispersion. Mechanical dispersion can provide colonies of "perfect" morphology, as it permits one to selectively pick undifferentiated colonies, or even undifferentiated parts from differentiated and overgrown cultures, but it is time-consuming and doesn't yield large numbers of cells, thus limiting expansion of the hES cell lines and experimental design. Nevertheless, this procedure is invaluable at the early stages of derivation, or as a means of producing more homogeneously undifferentiated plates of cells for expansion or for adaptation to trypsin. It is also a tool for a "rescue operation" in critical situations, when the success of salvaging a few colonies means saving a new hES cell line. When hES cell lines recover from freezing with low rates (some providers can only ensure the viability of 1% or less), or show aneuploidy (less than 100%), they can be "rederived" using mechanical passaging.

In new derivations or established cultures it is better to disperse colonies before they grow in contact with each other and prior to signs of differentiation, such as becoming multi-layered (Figure 41-2). The dispersed colonies may be left in the same well or moved to a new well. If there are only a few colony pieces (1–5) they should be placed near to each other, but with enough space to permit growth. When the cells are grown on a 35 mm dish, it is usually sufficient to disperse 50–100 average-sized colonies to populate a new 35 mm plate. In one to two days it may be necessary to disperse some of the larger colonies, leaving the pieces in the same well. Usually, mechanical passaging needs to be done every five to six days, but several larger colonies may need to be dispersed daily.

Materials Needed

Flame-pulled Thin Capillaries

We use an alcohol or gas burner to pull capillaries from glass Pasteur pipettes (pre-sterilized). The capillaries are broken by hand into angled tips (the shape of a hypodermic needle). The diameter of the capillaries may vary, but the best results are achieved when it is about 10–100 ES cells diameter, this is how large the colony pieces are going to be. The choice of a diameter depends on the operation: for instance, to do initial dispersion of an ICM outgrowth or to target undifferentiated parts of a colony, a diameter of 10–30 cells would be used, and to quickly harvest a large number of colonies from a good quality culture, a 50–100 cell capillary diameter would work the best.

Mouth-controlled Suction Device

Similar to a mouth pipette used for embryo transfer, this device consists of a mouth piece (Meditech International, cat. # 15601 P), rubber tubing, and a 0.22 μm syringe filter with a rubber tubing adapter for the capillary. It provides high precision in all manipulations for colony dispersions. Alternatively, a "Stem Cell Cutting Tool" (available from Vitrolife, Sweden) for picking colonies can be used instead of a mouth-controlled device and glass capillaries.

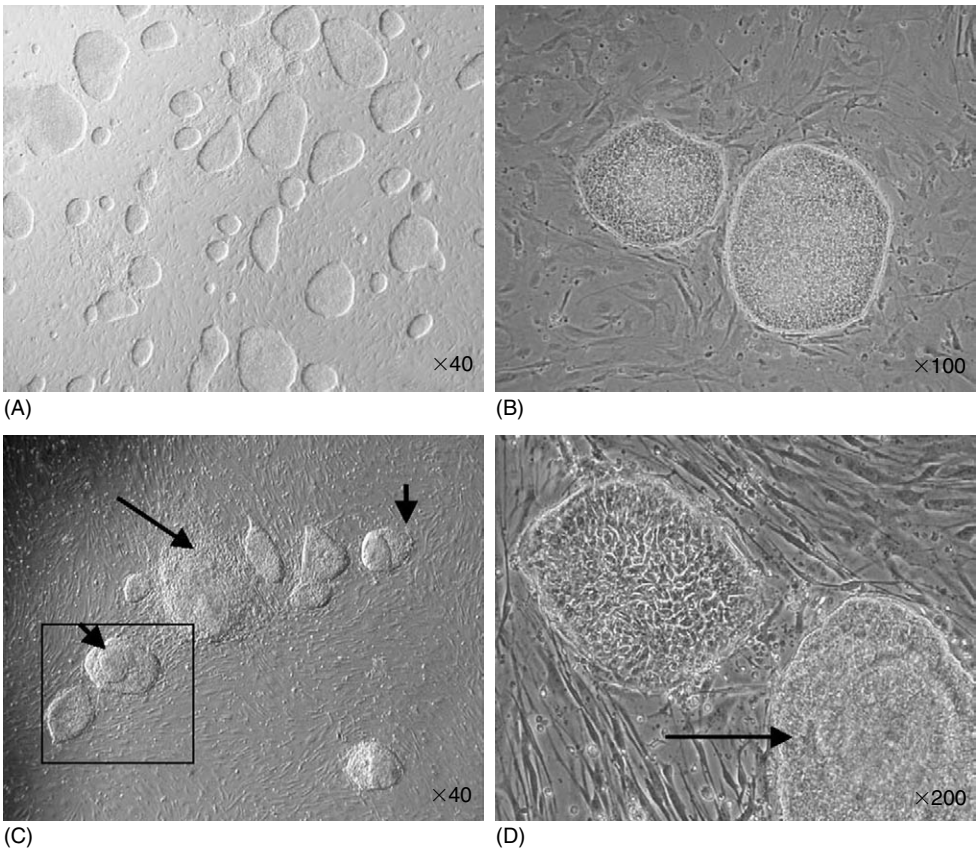


FIGURE 41-2 Morphology of mechanically dispersed hES cells. Colonies on panels (A, B) show no signs of differentiation. Note that the cells are small, tightly-packed, and the colonies have sharp borders. In (C) long arrow points to a very-differentiated colony that should not be dispersed, short arrows show partially-differentiated colonies which can be dispersed, and may produce undifferentiated colonies at the next passage. (D) Higher magnification of the framed colonies on (C), shifted 90º. The arrow points to a partially-differentiated colony, which has become multi-layered in the middle. The other colony is undifferentiated and is similar to colonies shown on (A, B).

Preparation of Primary Mouse Embryo Fibroblast Plate Receiving Mechanically Dispersed Colonies

1. When dispersing the initial ICM outgrowth, change the medium on a 4-well plate of Mitomycin C treated PMEF to hESC derivation medium the night before the derivation day, to let it become conditioned by the PMEF, final volume of 250 µl. Alternatively, instead of conditioning it overnight, the derivation medium can be supplemented with 30% of hESC-conditioned medium. To collect hESC-conditioned medium, add medium to a sub-confluent culture of hES cells with good morphology (see Figure 41-2A for an example of colony density and morphology), leave for 24 hours, collect medium, filter and store for two to three days at 4ºC. At early derivation stages, when the colony growth is slow, change ⅔ of the medium every two to three days to keep it conditioned at all times. As more colonies appear, change ⅔ of the medium daily and increase volume to 500 µl/well of a 4-well plate.

2. For established cultures, remove ⅓ of the medium from growing culture, put on new PMEF dish and add ⅔ volume of fresh medium. Change ⅔ of the medium daily.

Mechanical Dispersion

The procedure is similar to vacuuming. Gentle dispersion of the colonies is achieved by simultaneously cutting off the pieces with the angled end of the capillary, very lightly moving them off and sucking them in. With the opening of the capillary positioned horizontally to the bottom of the dish, begin moving from the sides in towards the colony center, chopping off and gently sucking in each piece. The light suction helps to detach the colony parts, and is applied at all times, as you move from the periphery of the colony, collecting the colony parts. If the whole colony is coming away from the monolayer in one piece, it is probably differentiated and should be discarded.

When the desired number of colonies is dispersed, blow out the pieces into the same plate (like for the first ICM dispersion), or into a freshly prepared plate. To avoid having all the colonies stuck to each other in the center of the plate, move the plate gently from side to side, do not swirl. (Figure 41-2 shows examples of mechanically passaged cultures, pointing out parts of colonies that have differentiated and should be avoided when passaging the culture).

DERIVATION OF HUMAN EMBRYONIC STEM CELLS

It is clear there are many factors which are not fully understood, that influence whether an isolated ICM will produce an hES cell line. Some of the factors to consider are: at what stage the embryo was frozen and by what procedure; the length of time the embryo must be in culture to generate a blastocyst; the culture conditions; and the quality of both the ICM and the trophectoderm. The determination of when an embryo is ready for immunosurgery must be determined empirically, usually occurring between d5 and d7. Any embryo that has undergone cavitation and has relatively intact trophectoderm is a candidate for immunosurgery.

Immunosurgery

The process of immunosurgery was performed essentially as described in Solter and Knowles, 1975. It involves removing the zona pellucida with Acid Tyrode's solution, incubating the embryo in an antibody that binds to the trophectoderm and preferably not to the ICM cells (especially important for the embryos with non-intact trophectoderm), and then lysing the trophectoderm cells with complement. The dead cells that surround the ICM are removed by passing the ICM through a narrow capillary. The isolated ICM is put on a prepared PMEF (in microdrops or 4-well plates) for further growth and dispersion.

Materials Needed

- Acid Tyrode's (Specialty Media, cat # MR-004.D).
- Rabbit anti-human red blood cell (RBC) antibody (purified IgG fraction, Inter-Cell Technologies, Hopewell, New Jersey, cat. # AG 28840).
- Aliquoted and stored at −80ºC, freshly diluted 1:10 in hES C Derivation Medium.
- Complement (Sigma, cat # S1639).
- Aliquoted and stored at −80ºC, freshly diluted 1:10 in Derivation Medium.
- Capillaries for embryo transfer.
- Thinly drawn capillaries (approximately the diameter of the ICM) for the trophectoderm removal,

Prepared Mitomycin C Treated Primary Mouse Embryo Fibroblast Plates

For the initial ICM outgrowth, change the medium on a 4-well plate of Mitomycin C treated PMEF to hESC derivation medium the night before the immunosurgery, to let it become conditioned by the PMEF, final volume of 250 µl.

Alternatively, instead of conditioning it overnight, the derivation medium can be supplemented with 30% of hESC-conditioned medium. To collect hESC-conditioned medium, add medium to a near-confluent culture of hES cells with good morphology (see Figure 41-2A for an example of colony density and morphology), leave for 24 hours, collect medium, filter, and store for two to three days at 4ºC.

Microdrops under oil can also be used to minimize the volume of the medium conditioned by the colony growing from an ICM well. To prepare a plate with microdrops first, make gelatin drops on a 60 mm tissue culture dish, and incubate for 30 minutes in a cell culture incubator. Aspirate each drop and immediately add to "footprints" PMEF suspension, 1 million cells/ml. Overlay with embryo-quality light mineral oil. Change the medium the next day to derivation medium, by carefully aspirating the medium from each drop under the dissecting microscope, and replacing with growth medium (a P200 pipette can be used); repeat once or twice to get rid of all PMEF medium.

Immunosurgery Procedure

1. Each embryo is processed separately. A dish is prepared with a series of 30 µl microdrops, three for each step: Acid Tyrodes, anti-human RBC antibody, complement, and three drops of derivation medium for each of the washes between the steps. The drops are covered with embryo-tested mineral oil, and are equilibrated in the CO_2 incubator for 60 minutes.

2. Under the dissecting microscope, transfer the embryo into the first Acid Tyrode's drop for a quick (1–2 seconds) wash, and then move into the second. Watch the embryo closely, and as soon as the zona pellucida thins and is nearly digested, move the embryo into the next series of hES cell media drops. Move the embryos through the first antibody drop into the second and third drops, and place the dish into the incubator for 30 minutes.

3. Transfer the embryos through three drops of Derivation Medium, and through three drops of the complement solution as described above; incubate in the last drop of complement in the CO_2 incubator for 15 minutes, and check for any "bubbling" trophoblast cells. If no cells show signs of lysis or if only a few cells are "bubbling," continue the incubation and recheck in five minutes. The embryo should be transferred to the drop of Derivation Medium as soon as all trophoblast cells are lysed or if no new "bubbling" cells appear after rechecking; the total incubation in complements should not exceed 30 minutes.

4. Gently pass the embryo through the opening of a thinly drawn capillary (about the diameter of the ICM), the lysed trophectoderm cells should detach after 1–2 passes.

5. Wash the ICM in the drops with Derivation Medium, and place into the prepared well of a 4-well plate. The ICM should attach within 24 hours.

Inner Cell Mass Dispersion

At the early stages of derivation, we recommend doing the first dispersion as soon as at least 2–3 colony pieces can be

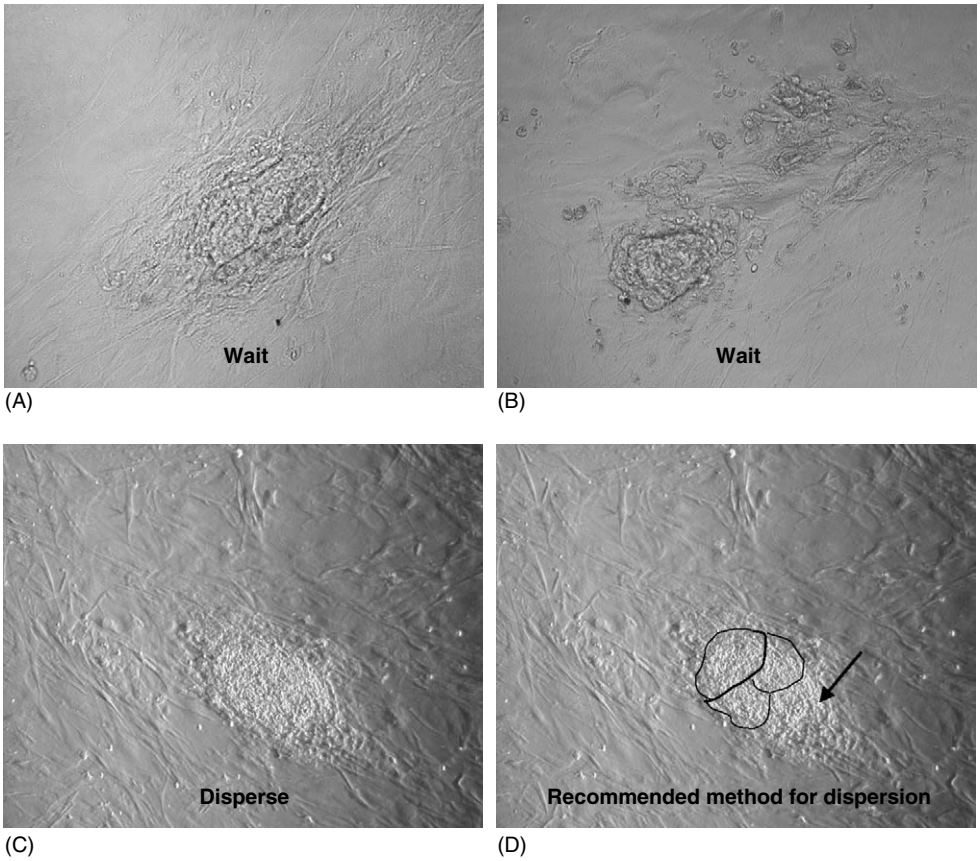


FIGURE 41-3 Initial ICM outgrowth.The initial outgrowth of the ICM rarely shows typical ES colony morphology, and often includes many differentiated-looking cells. When no obvious ES cell-like colonies large enough for dispersion can be located, more time is required before the first colony dispersion can be done. (A, B) The initial ICM outgrowth of two future hES lines. At this stage, when the dispersion is attempted, the outgrowth and the PMEF monolayer come up together, so it is better to wait longer before dispersing. (C) The ICM is ready for dispersion, when a colony of ES-like cells is formed which is large enough for dispersion into several pieces, leaving 20–50% of the outgrowth on the original place for future regrowth. (D) Dotted lines transcribe the number of pieces recommended for the dispersion of this colony. A narrow capillary is used and a small part of colony is left untouched (arrow). Magnification × 100.

obtained from the initial outgrowth (see Figure 41-3). The dispersed colonies may be left in the same well or moved to a new well. If there are only a few colony pieces (1–5) they should be placed close to each other, but with enough space to permit growth. It is better to disperse colonies before they grow in contact with each other and prior to signs of differentiation, such as becoming multilayered. When the colony growth is slow, change ⅔ of the medium every two to three days to keep it conditioned at all times. As more colonies appear, change ⅔ of the medium daily and increase volume to 500 μl/well of a 4-well plate.

Even when an original colony looks differentiated or comes off as a single piece, when replated, it usually gives an outgrowth of hES cells. When doing the initial dispersion, a part of the original colony should be left untouched as a back-up, especially if the picked pieces are transferred into a new well. Expect it to grow back in 1–2 days, and the new outgrowth can be picked and recombined with previously picked cells. Multiple harvests can be obtained from the initial outgrowth. It is critical at this stage to expand the number of colonies slowly and steadily. See Figure 41-4 as an example of the length of time between dispersions and appearance of the cultures during the process of derivation. In this case, no immunosurgery was done because the trophectoderm was not sufficiently intact.

MAINTENANCE OF ESTABLISHED HUMAN EMBRYONIC STEM CELL CULTURES

Usually, once a steady growth of colonies is reached, use of the hESC Derivation Medium is discontinued and the cultures are maintained in hESC Growth Medium. For established cultures, replace ⅔ of the medium volume with fresh medium daily; do not allow the medium to turn yellow. Cultures should be expanded gradually, by the progression from relatively sparsely populated 4-well dishes to confluent 4-wells to 35 mm dishes. Throughout the process, the cultures should be observed daily, differentiated colonies removed and undifferentiated colonies dispersed as necessary

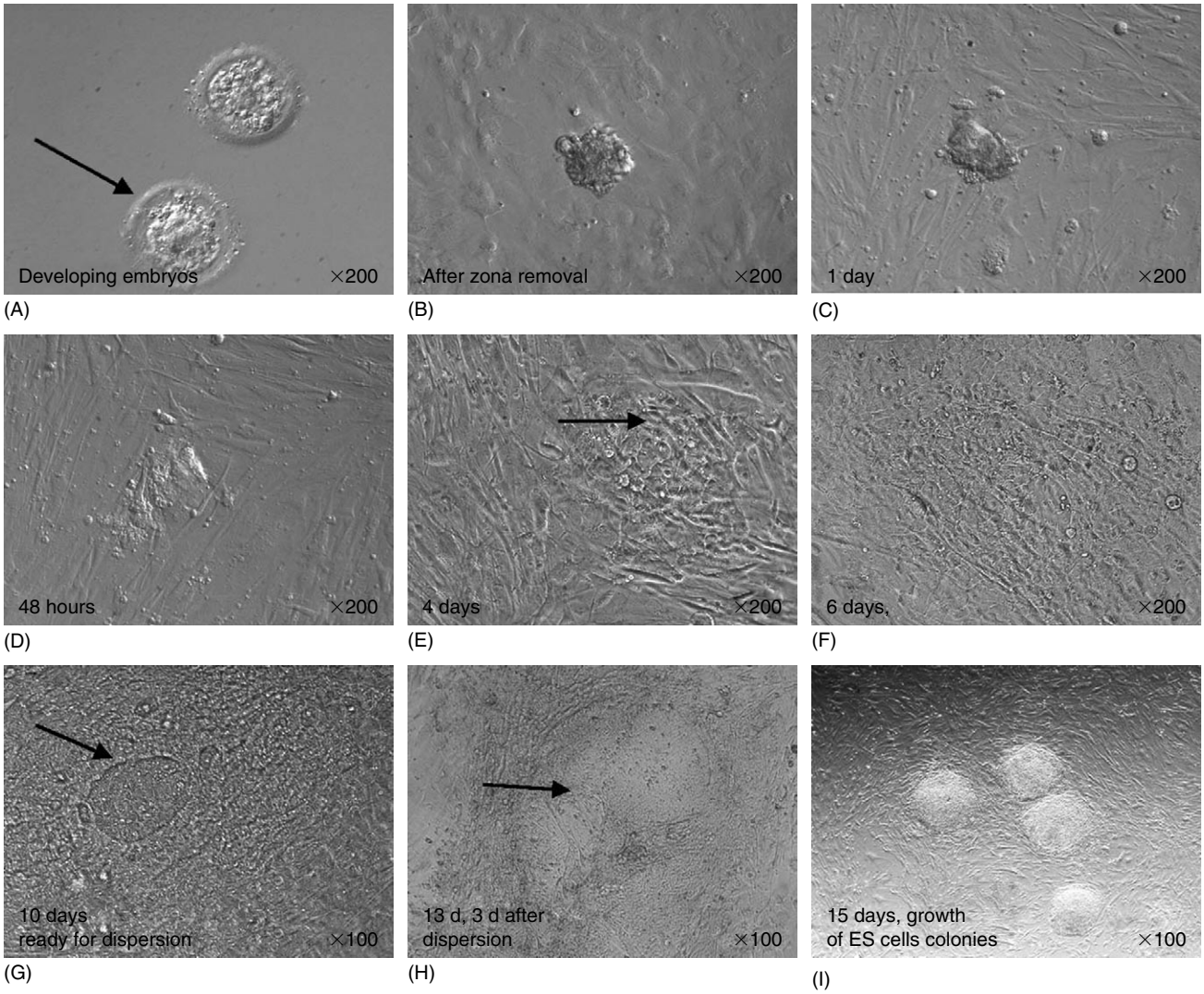


FIGURE 41-4 Early stages of hES cell derivation. A blastocyst of poor quality, grade 3, arrow in (A) underwent the removal of the zona pellucida (B), and next day after being plated on the PMEF monolayer has attached (C). In 48 hours after plating the ICM appears smaller, possibly due to some cell death (D), and in four days after plating an outgrowing group of cells is visible with some small cells in the middle (E, arrow) which become less visible two days later (F). Ten days after plating (G) a small colony of ES-like cells (arrow) has formed within the large group of differentiated-looking cells, and is now large enough for dispersion. Original ICM outgrowth (H) two days after it was dispersed; note regrowth of small ES-like cells in the cleared area (arrow) which are ready for a further dispersion. (I) Formation of ES cell colonies from the recombined first and second dispersion.

(see Figure 41-5 for examples of approaches to be used for dispersion of colonies with different morphologies).

By the time the cells are growing on a 35 mm dish, it is usually sufficient to disperse 50–100 average-sized colonies to populate a new well. In one to two days it may be necessary to disperse some of the larger colonies, leaving the pieces in the same well. Usually, mechanical passaging needs to be done every five to six days, but several larger colonies may need to be dispersed daily.

Adaptation of Human Embryonic Stem Cells to Trypsin

Our experience with trypsinization of over 30 hES cell lines demonstrates that, after the initial adaptation of the lines to trypsin, this procedure can be robust and yield large quantities of hES cells which exhibit all the properties of pluripotential cells. Trypsinized cells retain undifferentiated colony morphology, express characteristic molecular markers, i.e., Oct-4, alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, differentiate into three germ layers *in vitro*, and into teratomas *in vivo*, and maintain normal karyotypes.

Newly-derived hES cells may be successfully passaged with trypsin as early as passage 2–3 from a 4-well plate. However, trypsinization is not always successful, and several attempts may be necessary before the cells are adapted to trypsin. We recommend always keeping a back-up well of mechanically passaged cells. It has been shown (Draper *et al.*, 2004; Mitalipova *et al.*, 2005; Chan *et al.*,

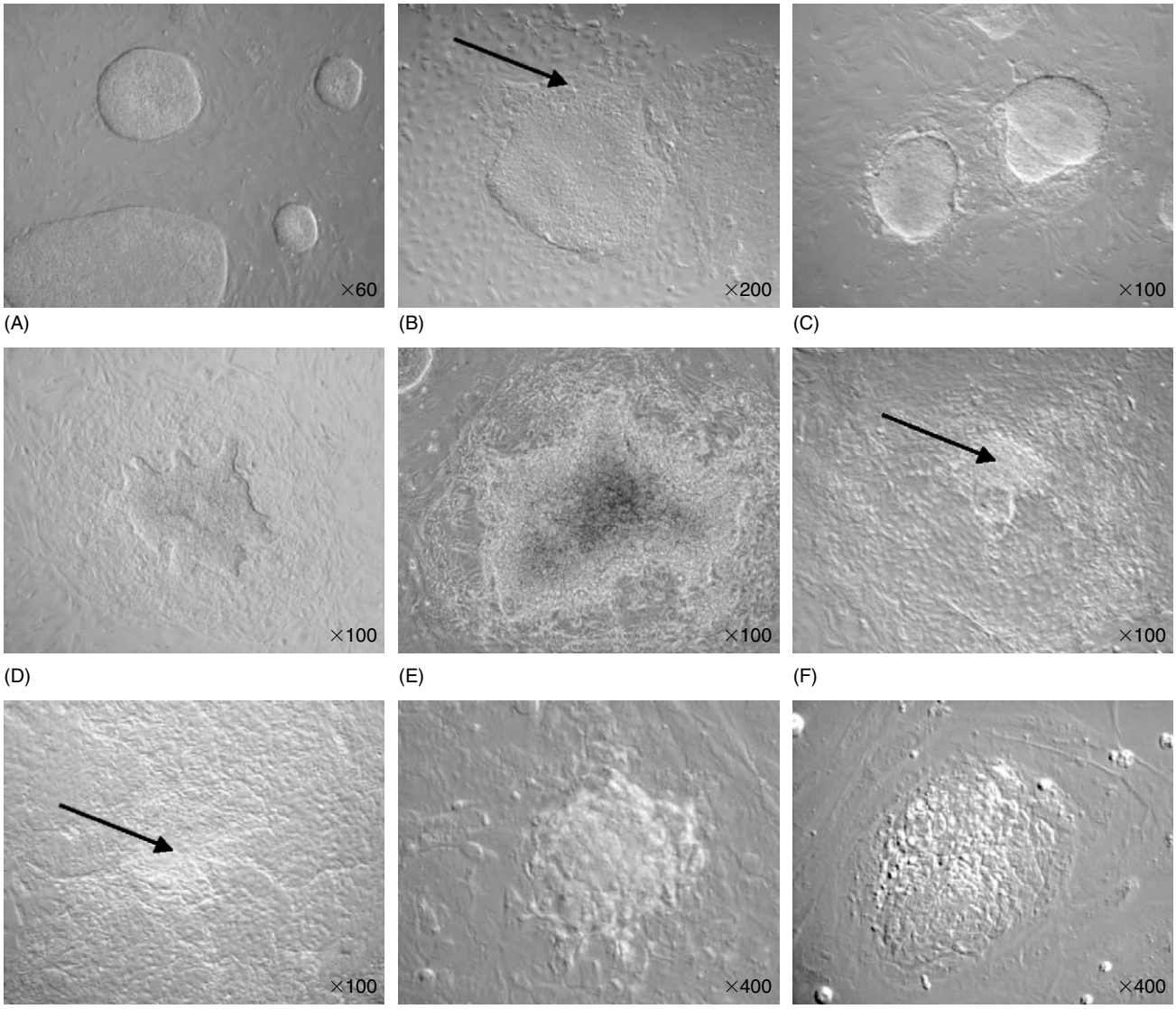


FIGURE 41-5 Evaluation of hES cell colony morphology. Various types of morphology of hES cell colonies encountered at early stages of derivation when mechanical dispersion can be the tool of choice to selectively pick undifferentiated colony parts. (A) All colonies are undifferentiated and can be mechanically passaged. (B) Colony which has a few signs of differentiation (arrow) and is surrounded by differentiated cells; the undifferentiated part is easily separated from surrounding differentiated cells. (C) Partially-differentiated, multi-layered colonies where the centers are thickened and yellowish in color can be mechanically dispersed into several small pieces, but may result in both differentiated and undifferentiated colonies. (D, E, F) All these colonies are more extensively-differentiated, a thin layer of differentiated cells covers them like a veil. They can be cut into pieces through the top cell layer and passaged, may yield undifferentiated colonies. (G, H, I) These colonies are badly-differentiated; the arrow on (G) shows a group of undifferentiated cells within the large differentiated area. If this one must be saved, one should wait for a few days for this group to increase in size.

2008) that aneuploidy can develop in long-term cultures, passaged enzymatically, therefore we recommend periodical karyotyping, and if aneuploidy is apparent, an earlier frozen stock should be expanded, or the line can be re-established by mechanical passaging if only a fraction of the cells appear aneuploid.

The safest approach is to begin with a sub-confluent 35 mm plate of colonies with good morphology. Mechanically pick 50–100 colonies and transfer into a new well as a back-up. Differentiated colonies may be removed mechanically prior to trypsinization. Trypsinize the remaining colonies in the original well, and plate into the same diameter well. The cells should be ready for the next split in five to seven days (Figure 41-6). For second trypsinization, split 1:3. After this step the cells can usually be trypsinized routinely without problems, but a mechanical back-up should always be maintained until the cells are frozen, and the test vials are successfully thawed.

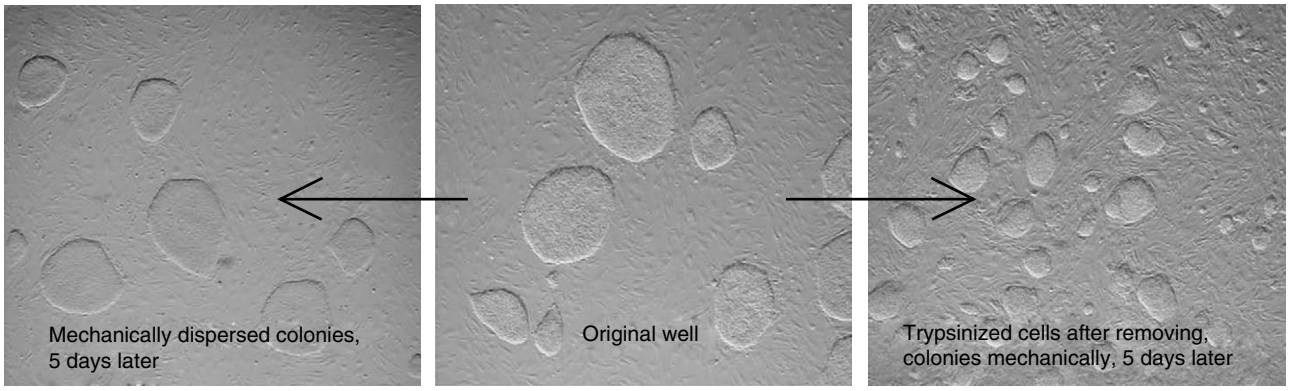


FIGURE 41-6 Adaptation of mechanically passaged hES cells to trypsin. After five days of growth, colonies were mechanically dispersed and transferred to a fresh plate of PMEF (A). Morphologically they are very similar to the original plate (B). The remaining colonies in the original plate, which were passaged with trypsin and plated onto the same diameter plate (C) show actively-growing colonies, and will probably be ready for passaging in one to two days.

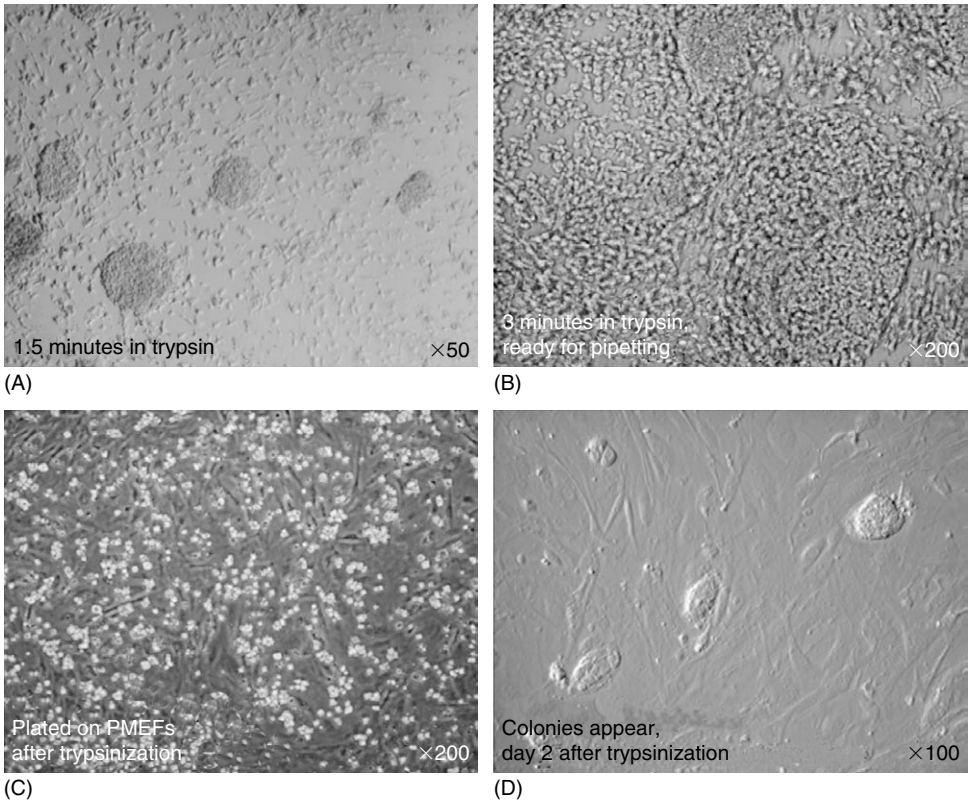


FIGURE 41-7 Passaging by trypsinization. (A) 1.5 minutes in trypsin, the PMEF look shrunken and the hES colonies have loosened a little. (B) 3 minutes in trypsin at higher magnification: the colonies compacted; this is a good time to begin pipetting. Please, note that depending on the density of the colonies, days after passaging, and the degree of differentiation, the time required to reach this stage may vary and needs to be worked out empirically. (C) Suspension of hES cells after replating onto fresh PMEF. Note the majority of the cells are in small cell aggregates. (D) Small colonies begin to appear on day 2 after plating (at 1:3 ratio). The time when the first colonies are seen may vary, depending on the splitting ratio and the size of the cell aggregates.

Trypsinization

Generally, hES cells recover from trypsinization better when not dispersed to single cells, but remain as small cell clumps of approximately 5–20 cells. The procedure works best when hES cell colonies are dispersed by a combination of enzymatic digestion and pipetting; we do the pipetting before the PMEF monolayer and the colonies turn into a single-cell suspension. The time in trypsin required for the cells to detach varies, depending on the hES cell density, degree of differentiation, age of the culture, temperature of trypsin, etc. Therefore, instead of providing a fixed incubation time in trypsin, we recommend checking the appearance of the hES culture under the microscope, and empirically working out the best incubation time for each plate (see Figure 41-7).

1. Warm trypsin in a 37ºC water bath; keep warm until ready for procedure.

2. Rinse the cells with PBS, Ca^{2+}-, Mg^{2+}-free, twice (1–2 ml per 35 mm dish).

3. Add 1 ml of trypsin to each 35 mm dish. Incubate in the hood at room temperature for several minutes, usually 2–5, frequently checking the cells under the microscope. The cells are ready for mechanical dispersion when the PMEF begin to shrink; the colonies should round up but remain attached. Some cells may begin to detach and float (Figure 41-7A, B).

4. Prepare a centrifuge tube with 10 ml of warm PMEF medium. (NOTE: It is necessary to use PMEF medium to inactivate the trypsin, because our hESC medium is serum-free.) Tilt the plate and begin to gently pipette the trypsin solution up and down with a 1 ml pipetteman (Gilson type), pouring it over the cell monolayer at an angle. Properly-digested cells should detach easily, leaving visible clear gaps in the monolayer, where the trypsin solution was poured. If no such gaps appear, leave it for another 1–2 minutes and test again. Expect the monolayer to detach after several repetitions. Usually, on cell cultures less than five-days-old, you should be able to completely disperse the monolayer, but if the culture is older or very dense, there may be some undigested material which can be discarded. Usually, it takes 5–10 pipetting strokes to break the colonies into small clumps of cells (see Figure 41-7C for approximate clump size). Extensive pipetting should be avoided. Using a cell culture pipette in place of an automatic pipettor will result in larger cell clumps, and thus may require longer time in trypsin.

5. Transfer the trypsinized cell suspension into the prepared centrifuge tube; centrifuge for five minutes at 160 g.

6. Aspirate the medium, and resuspend the pellet in hESC medium, again avoiding extensive pipetting to preserve small cell aggregates, and replate at the desired ratio. The colonies should become visible in one to two days, depending on the splitting ratio and the clump size (Figure 41-7D).

Although hES cultures passaged with trypsin can be maintained in an undifferentiated state, if conditions are unfavorable due to changes in media/reagents quality, too high or low splitting ratio, or problems with PMEF quality, the cultures can have a degree of differentiation which should be evaluated prior to the next trypsinization (see Figure 41-8).

Freezing Human Embryonic Stem Cells

Many of the established hES cells have low recovery rates upon thawing, as low as 0.1–1%. This may be due to the method of passaging the cells. Mechanical picking or using collagenase dispersion usually results in large cell aggregates, which presumably do not get cryopreserved as effectively as smaller clumps. Trypsinized cells in our

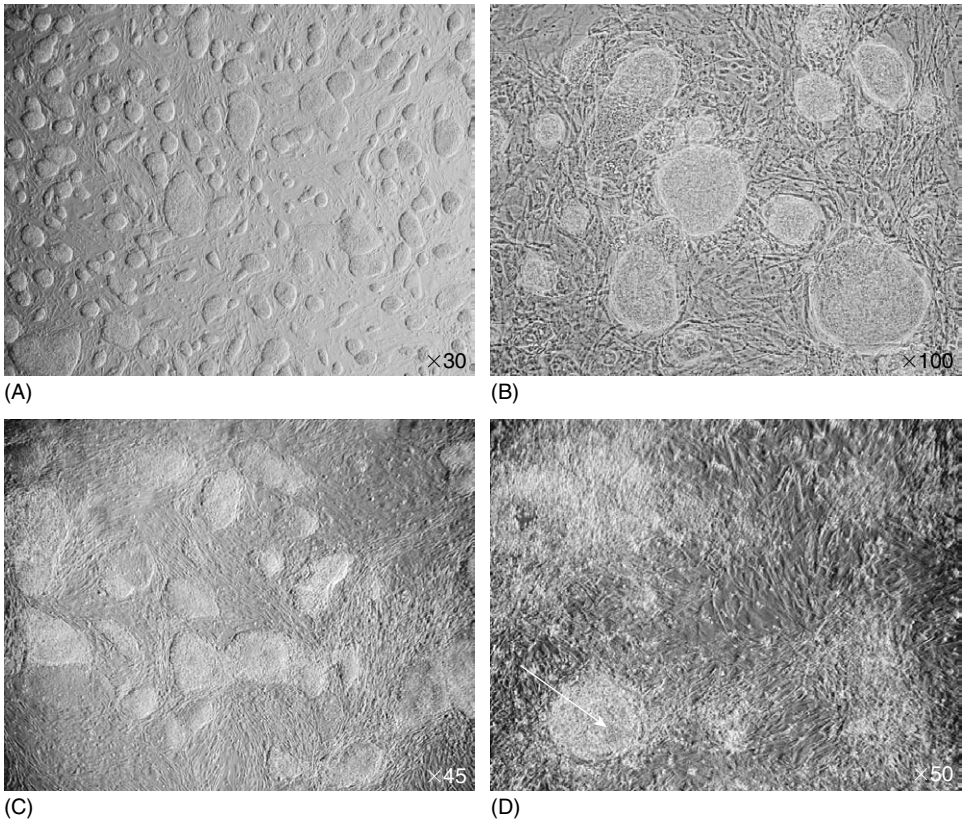


FIGURE 41-8 Evaluation of hES cell culture in preparation for the next trypsinization. (A, B) Mostly undifferentiated hES cells ready for trypsinization. (C) The colonies are a little overgrown and show signs of differentiation, but can still be safely passaged with trypsin. (D) Badly differentiated hES cells, an arrow points to a colony, which can still be rescued by mechanically picking colony pieces and passaging.

laboratory have a recovery rate of about 10–20% or higher, and do not require more complicated procedures like vitrification (Figure 41-9).

Freezing Medium

The best recovery rate was observed in freezing medium consisting of 90% FCS, 10% DMSO. However, Oct-4 expression in the thawed cells was lower than in cells frozen in hESC Growth Medium with 10% DMSO. Nevertheless, by the next passage, the expression and distribution of Oct-4 and other markers of undifferentiated cells were indistinguishable between these two freezing conditions. We routinely use the 90% FBS, 10% DMSO medium.

Freezing Protocol

Select a high-quality confluent culture with good morphology for freezing. We also recommend taking a picture of a sample field, and staining for molecular markers characteristic of undifferentiated hES cells for future reference.

Materials Needed

- Freezing medium: 90% FBS, 10% DMSO.
- Cryovials, labeled with the line, passage number, date.
- Cryovial rack (rack with ice reservoir by Corning).
- Styrofoam rack from packaging for 15 ml centrifuge tubes.
- −80ºC freezer.

1. Trypsinize the cells; centrifuge in PMEF medium (see above).

2. Resuspend the pellet in cold freezing medium. We recommend freezing one confluent 35 mm plate per vial in 0.5 ml of freezing medium. Work quickly, and keep the cells on ice after addition of the freezing medium.

3. Aliquot cell suspension into pre-chilled freezing vials and sandwich the vials between two Styrofoam racks; tape to prevent the two racks from separating and transfer to a −80ºC freezer overnight. Transfer cryovials to liquid nitrogen for long term storage.

Thawing Human Embryonic Stem Cells

Thawing hES cells is a relatively simple procedure. The main rule to follow is to do everything quickly.

Preparation

1. Prepare Mitomycin C-treated PMEF a day before thaw.

2. Make Thawing Medium. We use 70% hESC Growth Medium, supplemented with 20 ng/ml hLIF and 8 ng/ml bFGF with 30% hESC or PMEF-conditioned medium.

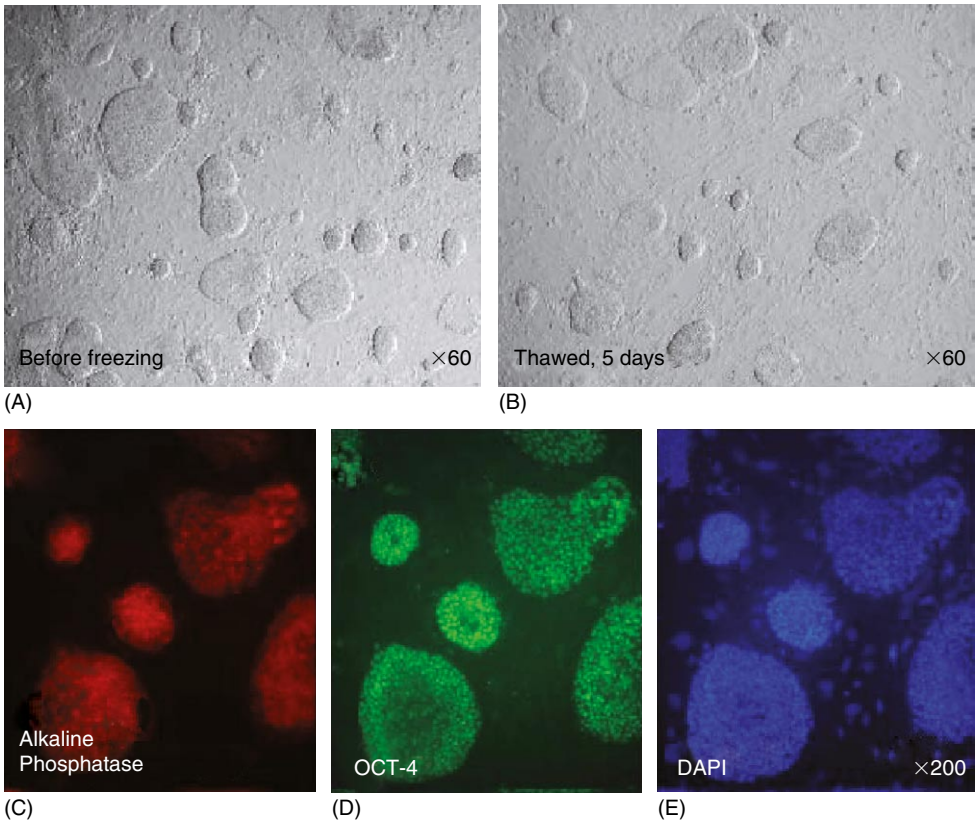


FIGURE 41-9 Freezing and thawing of hES cells. The top panel shows the approximate density and morphology of the colonies of hES cells at freezing (A). When thawed onto the same diameter plate, the colonies should be ready for the next split in five to seven days (B). Thawed hES cells show high expression of Oct-4 and alkaline phosphatase (C, D).

3. Change the medium on the PMEF plate to the Thawing Medium; equilibrate in the CO_2 incubator for one hour. For 35 mm plates use 1.5 ml, for 4-well plates use 0.5 ml medium per well.

4. Prepare a 50 ml conical tube with 10–15 ml of warm hESC growth medium.

Thawing

1. Thaw the vial(s) in a 37ºC water bath, constantly agitating, while ensuring that the neck of the vial is above the water level. Check the content of the vial in about 40 seconds, and after that at 10 second intervals, until only a small piece of ice remains.

2. Quickly spray the vials with 70% isopropanol then, using a 1 ml pipetteman, add warm hESC medium to the contents of the vial drop-wise with gentle agitation. Do it quickly, but very gently. Immediately transfer the contents into the prepared 50 ml tube with warm hESC medium centrifuge at 160 g for five minutes.

3. Remove the medium completely, not touching the pellet.

4. Add 0.5 ml hESC Thawing Medium; gently resuspend the cells using a 1 ml pipetteman (2–4 repetitions), transfer to prepared PMEF plates with equilibrated hESC Thawing Medium. Spread the cells evenly throughout the well by moving the plate several times in two directions, at 90º to each other, avoid swirling.

5. Check the cells the next day; if there are many dead cells or the medium has changed color, change ⅔ of the medium, otherwise, don't change it for another day.

6. The colonies usually begin to appear in three to four days, and can be ready for splitting in five to ten days (Figure 41-7).

Human Embryonic Stem Cell Quality Control

Although morphology of hES cells is often used for evaluating the quality of the culture and its readiness for passaging or freezing, this criteria alone cannot be used for assessment of the ES cells pluripotency. Staining for the expression of Oct-4 or alkaline phosphatase, even in colonies of "perfect" morphology, can result in one or both of these markers appearing in the cells only at the periphery of the colony. It is important, therefore, to regularly assess the cells by analyzing the expression of markers of pluripotent cells. We look at Oct-4, Nanog, SSEA-3, SSEA-4, TRA 1-60, and TRA 1-81 by immunostaining, or perform an enzyme assay for alkaline phosphatase. The procedures for such assays and available antibodies are described elsewhere (Xu, 2006). Routine karyotyping performed either in-house or commercially provides an assessment of the degree of aneuploidy in hES cultures, and testing for differentiation potential is another important parameter to assess. In our laboratory we routinely perform differentiation of hES cells into retinal pigment epithelium and hemangioblast as a part of other ongoing projects, and if abnormal cell behavior, such as a low differentiation rate, is observed in differentiation assays, the cultures are discarded and fresh stock is thawed. Other differentiation models for hES cells can be used for such routine assessment of the quality of the culture as determined by the research goals.

GLOSSARY TERMS

Conditioned medium Medium left in contact with cultured cells, usually for a prolonged period of time.

Immunosurgery A method of removing the trophectoderm of a blastocyst using antibodies bound to the surface antigens of the trophectoderm and complement.

PMEF (primary mouse embryonic fibroblasts) A mixed population of cells derived from dispersed mouse embryos and cultured for a limited number of passages under conditions favoring the growth of fibroblasts.

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Chapter 42

Derivation and Differentiation of Human Embryonic Germ Cells

Michael J. Shamblott, Candace L. Kerr, Joyce Axelman, John W. Littlefield, Gregory O. Clark, Ethan S. Patterson, Russell C. Addis, Jennifer N. Kraszewski, Kathleen C. Kent, and John D. Gearhart

Embryonic germ (EG) cells are pluripotent stem cells derived from primordial germ cells (PGCs) that arise in the late embryonic and early fetal period of development. EG cells have been derived from several species, including mouse, pig, chicken, and human. Mouse, pig, and chicken EG cells have been demonstrated to contribute to experimentally-produced chimeric animals, including germline transmission in the latter two species. Furthermore, germline transmission of mouse and chicken EG cell derivatives have been demonstrated. Mouse and human EG cells can be differentiated *in vitro* to form embryoid bodies (EBs). Like EBs generated from embryonic stem (ES) cells, EG-derived EBs contain differentiated cells representing all three germ layers, as well as mixed-cell populations of less differentiated progenitors and precursors. These human EB-derived (EBD) cells are capable of considerable cell proliferation and express a variety of lineage-specific markers. Human EBD cell cultures have a normal and stable karyotype and normal patterns of genomic imprinting, including X-inactivation. Transplantation studies have demonstrated that human EBD cells can engraft into a variety of rodent tissues and can participate in the recovery of rats following motor neuron injury.

INTRODUCTION

Pluripotent stem cells have been derived from two embryonic sources. Embryonic stem (ES) cells were first derived from the inner cell mass of mouse pre-implantation embryos, and embryonic germ (EG) cells were initially derived from mouse primordial germ cells (PGCs). Subsequently, EG cells have been derived from chicken, pig, and human PGCs. Pig, chicken, and mouse EG cells have been demonstrated to contribute to experimentally-produced chimeric animals, including germline transmission in the latter two species.

Primordial Germ Cells

PGCs are the sole means of genetic transmission between parent and offspring, as they generate eggs and sperm. In many species, such as *C. elegans*, germ cells are segregated very early in development, during the first embryonic cleavages, and are marked by deposition of ribonucleoprotein P-granules. In mammals, the process occurs later in development, and seems to be directed more by extrinsic factors than by preprogrammed intrinsic differences. For example, in mice, cells that generate PGCs are located close to extraembryonic ectoderm during gastrulation. Rather than having a previously determined fate, cells in this location receive external signals to further differentiate into PGCs, as demonstrated by the observation that transplantation of cells from other parts of the epiblast to this region can take on a PGC fate. Several components of this signaling process have been identified. Initially, bone morphogenetic protein 4 (BMP4) and BMP8b are produced by extraembryonic ectoderm program cells from the epiblast to become extraembryonic mesoderm precursors or PGCs. Cells destined to become PGCs express higher levels of membrane protein fragilis than nuclear protein stella.

In the mouse, PGCs are visible as alkaline phosphatase (AP) positive cells at the base of the allantois at 7.5 to 8.0 days postcoitus (dpc). They begin to associate with the endoderm that is invaginating to form the hindgut at 8.5dpc. By 10.5dpc, PGCs are associated with dorsal mesenteries and are translocated to the genital ridges. The migration of PGCs is caused by both cellular migration and association with moving tissues. Throughout this migration, PGCs expand from approximately 130 cells at 8.5dpc to more than 25 000 at 13.5dpc. Once they arrive at the genital ridge, PGCs continue proliferating until they enter prophase of the first meiotic division. In males,

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entry into meiosis is inhibited by signals from the developing testis, blocking PGCs at G0 until after birth. In the absence of inhibitory signals, female PGCs undergo oogenesis. Although not as thoroughly studied, much is known regarding the migratory path of human PGCs, including their association with gut endoderm and migration into developing genital ridges.

PGCs do not survive well under standard tissue culture conditions, and are not pluripotent stem cells *in vivo* or *in vitro*. Early attempts to use various growth factors and feeder layers succeeded in prolonging their survival, but proliferation was limited. The combination of leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and c-kit ligand (KL, also known as stem cell factor, mast cell factor, or steel factor) proved to result in an immortal cell population, especially if the KL was presented in the transmembrane form by a layer of "feeder" cells (see the section Feeder Layer). Instead of simply encouraging PGC proliferation, these factors cause the normally solitary PGCs to congregate and proliferate as multicellular colonies, known as EG cells, and to gain pluripotency. Mouse EG cell lines have been derived from PGCs prior to migration around 8.0–8.5dpc, during migration at 9.5dpc, and after entry into the genital ridges between 11.5 and 12.5dpc.

The roles played by KL and the tyrosine kinase receptor for KL, c-Kit, in the *in vitro* derivation of EG cells from PGCs have parallels *in vivo.* c-Kit is expressed in PGCs, and KL is expressed along the PGC migratory pathway and in the genital ridges. The roles of KL and c-Kit in PGC survival were originally characterized through several mutations at their respective loci, *Sl* and *W*, which resulted in subfertile or sterile mice. PGCs are formed in homozygous mutant embryos of *W* and *Sl*, but mitosis is severely impaired, and the few PGCs that reach the gonad do not survive. KL is produced as a membrane-bound growth factor that can undergo proteolytic cleavage to generate a soluble form. Mice lacking the membrane-bound KL, but not the soluble form, maintain low PGC numbers and are sterile, suggesting that the membrane-bound form but not the soluble form is essential for PGC survival. The mechanism involved in KL-induced PGC survival has been shown to involve suppression of apoptosis. The c-kit receptor has also been shown to be involved in the adhesion of mouse PGCs to somatic cells *in vitro.* Other recent studies attempting to identify signaling pathways activated by KL binding to its receptor in mouse PGCs have shown activation of AKT kinase and telomerase.

In contrast to the embryologically early and relatively undifferentiated epiblast, PGCs arise late and have a specialized role during normal development. In this regard, it is somewhat surprising that exposure to three cytokines can convert PGCs to pluripotent stem cells *in vitro.* It is possible that the flexibility provided by extrinsic signaling during PGC specification, rather than intrinsic preprogramming, allows for this conversion.

Comparison to Embryonic Stem Cells

Both mouse ES and EG cells are pluripotent and demonstrate germline transmission in experimentally produced chimeras. Mouse ES and EG cells share several morphological characteristics, such as high levels of intracellular AP and presentation of specific cell-surface glycolipids and glycoproteins. These properties are characteristic of, but not specific for, pluripotent stem cells. Other important characteristics include growth as multicellular colonies, normal and stable karyotypes, the ability to be continuously passaged, and the capability to differentiate into cells derived from all three EG layers: endoderm, ectoderm, and mesoderm.

HUMAN EMBRYONIC GERM CELL DERIVATION

Although many combinations of cytokines and feeder layers have been evaluated, the standard practice for derivation of human EG cells remains similar to techniques developed for the mouse. As of 2003, approximately 140 human EG cultures have been derived in our laboratory using this general technique.

Initial Disaggregation and Plating

Gonadal ridges and mesenteries of week 5–9 post-fertilization human embryos (obtained as a result of therapeutic termination of pregnancies) are collected in 1 ml ice-cold growth media and rapidly transported to a sterile work space. The tissues are then soaked in calcium–magnesium-free Dulbecco's phosphate-buffered saline (DPBS) for five minutes, and then transferred to 0.1 ml trypsin–EDTA solution. The concentration of trypsin and EDTA is varied, such that at the earliest developmental stages, a gentler 0.05% trypsin–0.5 mM EDTA is used, and at later developmental stages, a stronger 0.25% trypsin–0.5 mM EDTA solution is used. The tissue is mechanically disaggregated thoroughly using a fine forceps and iris scissors. This process is carried out for 5 to 10 minutes at room temperature and then incubated at 37ºC for 5 to 10 minutes. This disaggregation process often results in a single-cell suspension and large pieces of undigested tissue. To stop the digestion, serum-containing growth media is added. The digested tissue is transferred to wells of a 96-well tissue culture plate that has been previously prepared with feeder layer (see the sections Plating an STO Feeder Layer Prior to Inactivation and Plating an STO Feeder Later after Inactivation). Usually, the initial plating occupies four to 10 wells of the 96-well plate. The plate is incubated at 37ºC in 5% CO_2 with 95% humidity for seven days. Approximately 90% of the growth media is removed each day, and the plate is replenished with fresh growth media.

Subsequent Passage of Embryonic Germ Cell Cultures

In the first seven days of derivation (passage 0), most human EG cultures do not produce visible EG colonies. Staining for AP activity demonstrates the presence of solitary PGCs, with either stationary or migratory morphology (Figure 42-1A, B). Often, colonies of cells that do not stain AP+ are seen (Figure 42-1D, E), as are small clumps of tissue remaining from the initial disaggregation (Figure 42-1C). After seven days, the media is removed, and the wells are rinsed twice with calcium–magnesium-free DPBS for a total of five minutes. Then, 40 ml of freshly thawed trypsin solution is added to each well, and the plate is incubated on a heated platform or in a tissue culture incubator for five minutes at 37ºC. As previously described, the trypsin solution is 0.05% trypsin–0.5 mM EDTA, 0.25% trypsin–0.5 mM EDTA, or a mixture of these two solutions. The important point at this stage is to facilitate the complete disaggregation of the STO cell feeder layer (see the section Feeder Layer), which can be a significant challenge. After the incubation in trypsin, the edge of the 96-well culture plate is hit firmly against a solid surface until the STO cells have completely lifted off the growth surface. This process can be aided by scraping the well and gently triturated. After the STO cells have been loosened, fresh growth media is added to each well, and the contents are triturated. This phase is critical to successful disaggregation of STO feeder layer and EG cells.

All subsequent passages are done as described. After 14 to 21 days (during passage 1 or 2), large and recognizable EG colonies will arise in some of the wells (Figure 42-2). At this point, wells that do not have EG colonies are discarded.

Several common problems occur during the passage of human EG cells. One observation is that the STO feeder layer will sometimes not fully disaggregate. This can be observed by the presence of large cell aggregates immediately following disaggregation. If this occurs with regularity, it is a sign that the trypsinization method is insufficient, or that the STO cells have become less contact inhibited and have overgrown during the seven days of culture. Another common occurrence is that the EG colonies do not fully disaggregate. The consequences of poor disaggregation

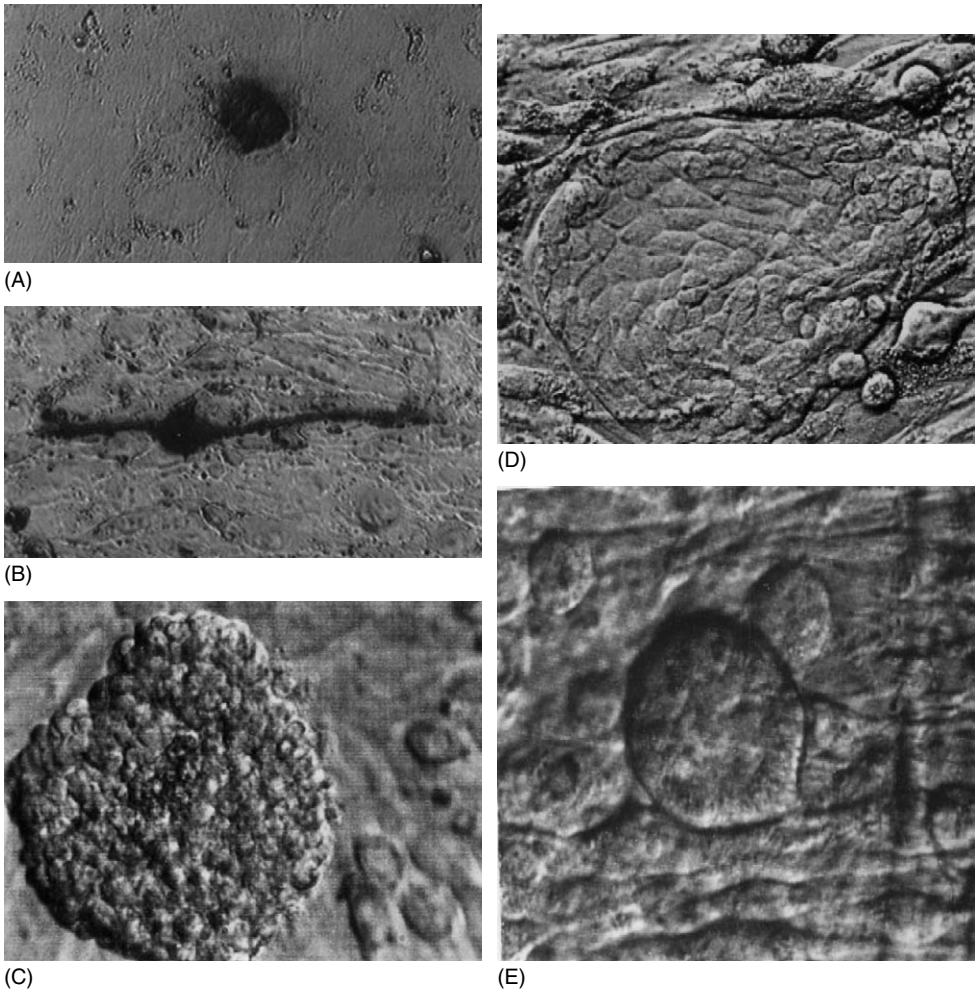


FIGURE 42-1 Cell morphologies seen in early passage human embryonic germ culture. (A, B) Alkaline phosphatase-positive (AP+) stationary and migrating primordial germ cells. (C) Multicellular piece of undisaggregated gonadal tissue. (D, E) Flat and round cell colonies that do not lead to human EG cells.

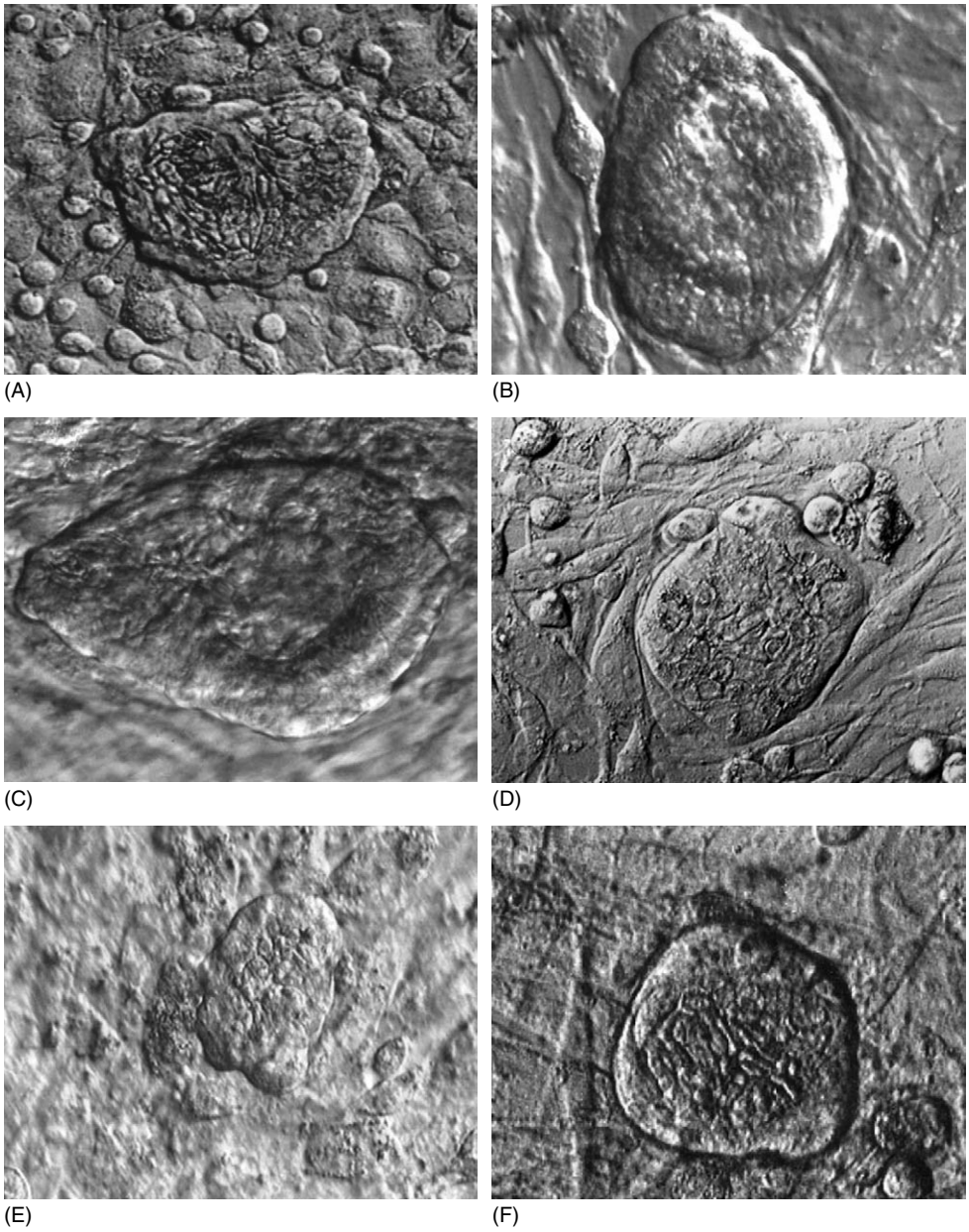


FIGURE 42-2 Human EG colonies growing on an STO cell feeder layer.

are that the large pieces differentiate or die, and fewer EG cells are available for continued culture expansion. Although much effort has been expended to find a solution to this problem, it remains the most difficult aspect of and challenging hurdle to human EG cell biology.

To gain some insight into this problem, a series of electron microscopic images were taken to compare the cell–cell interactions found in mouse ES, mouse EG, and human EG cell colonies. It is evident from these images that cells within the human EG colonies adhere more completely to each other than cells within mouse ES and EG colonies (Figure 42-3). It is possible that this tight association within the colony limits the access of disaggregation reagents. At this time, neither the nature of the cell–cell interactions nor an effective solution to this problem is evident.

Because of incomplete disaggregation and other intrinsic or extrinsic signals, many human EG colonies (10–30% per passage) differentiate to form three-dimensional structures termed EBs (see the section EB Formation and Analysis) or flatten into structures that are AP− and do no continue to proliferate (Figure 42-4). EG colonies that are more fully disaggregated go on to produce new EG colonies, and under the best circumstances, EG cultures can be expanded continuously for many months, and have routinely exceeded 20 passages. Inevitably, as large EG colonies are removed from the culture as a result of EB formation, the cultures become sparse, and are discontinued for practical considerations. Efforts employing standard dimethyl sulfoxide (DMSO) cryopreservation techniques have so far been unsuccessful.

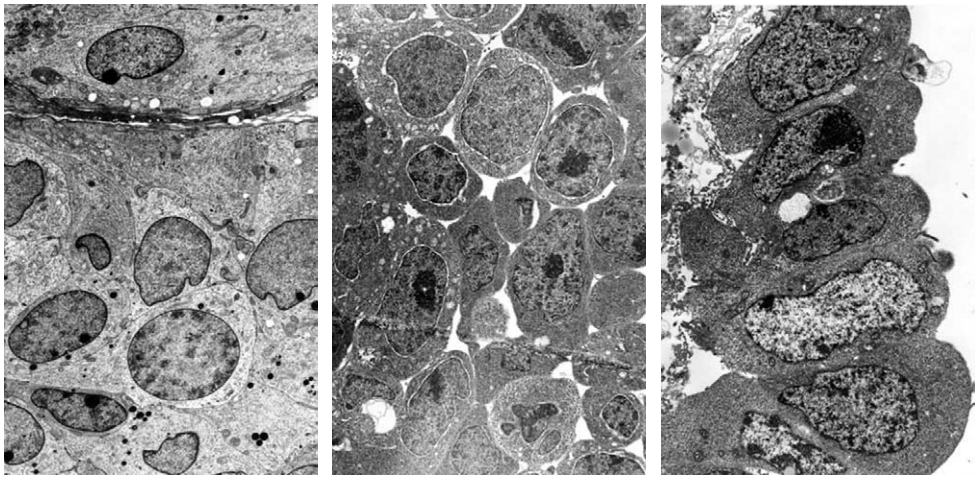


FIGURE 42-3 Electron micrograph of EG and ES colonies. (A) Human EG colony; (B) mouse EG colony; and (C) mouse ES colony.

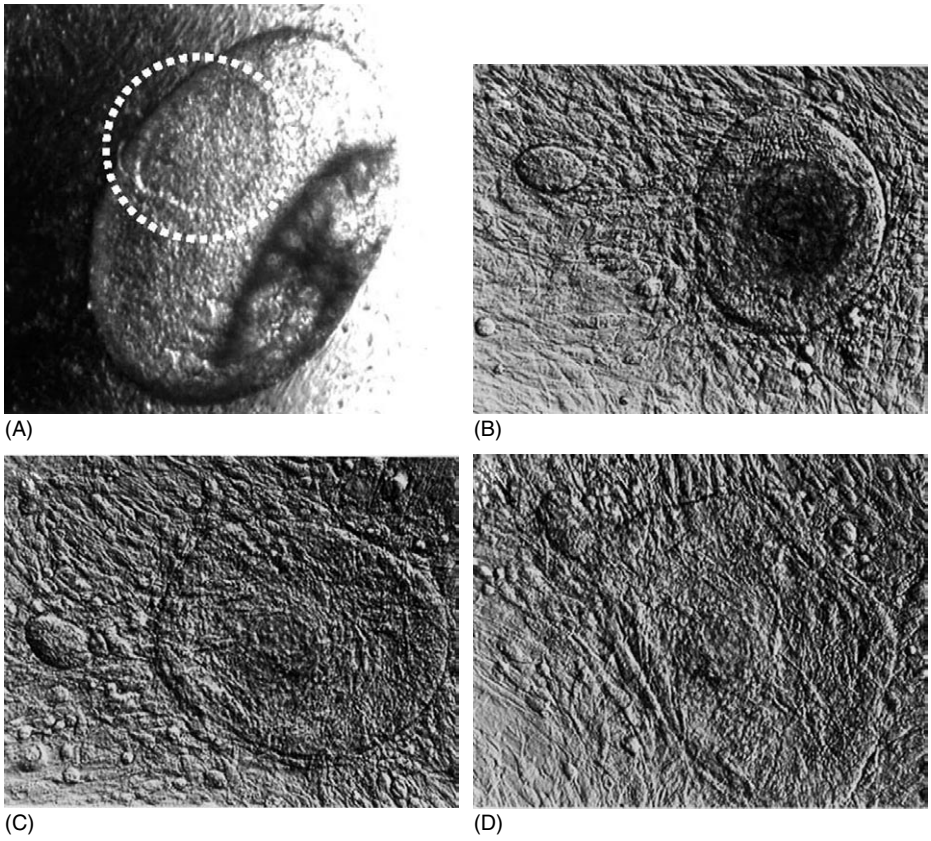


FIGURE 42-4 Differentiating human EG colonies. (A) Stereomicrograph of an EG colony with attached embryoid body (EB). The EG colony is circled. The diameter of the EB is approximately 0.5 mm. (B–D) Time-lapse study of EG cell flattening. (B) Four days after disaggregation, the small EG colony on the left results from more complete disaggregation. The large colony on the right is composed of a dark residual colony surrounded by new EG cell growth. (C) Five days after disaggregation, the large EG colony has begun to flatten. (D) Seven days later, the large EG colony has completely flattened and did not survive as a recognizable EG cell colony after disaggregation and replating. In a separate experiment, EBs and these flattened structures are shown to be largely AP−.

Feeder Layer

Unlike ES cells, EG cell derivation is highly dependent on a specific type of feeder layer. Mouse STO fibroblasts are a spontaneously transformed cell line from the Sandoz inbred mouse. They are thioguanine- and ouabain-resistant, features of historical interest, but not used in this context. STO cells have been used to derive mouse embryonal carcinoma (EC), ES, and EG cells, and to date, they are the only cell type capable of generating human

EG cells. The factor or factors provided exclusively by this cell type are not fully understood. However, the transmembrane form of KL is present on STO fibroblasts, but not on most other cell types evaluated in our laboratory.

Although STO is a clonal cell line, individual isolates vary greatly in their ability to support human EG derivation. This is further complicated by the known phenotypic variation of STO cells in continuous culture. Given the very limited supply of human tissue, it is prudent to screen STO cells for suitability prior to use. The most reliable screening method is to produce a number of clonal STO lines (by limiting dilution or cloning cylinder), and to evaluate them for their ability to support the derivation of mouse EG cells. The growth of existing mouse EG lines is not a sufficient method, as most mouse EG lines become feeder layer independent after derivation. Derivation of mouse EG cells is not a simple undertaking. In an effort to screen STO cells more rapidly, and to investigate the role of transmembrane KL, we have begun to screen STO cell lines by using an immunocytochemical method to detect KL. Once a supportive STO fibroblast line is identified, it should be immediately cryopreserved in several low-passage aliquots. One of these aliquots can then be expanded to provide many medium passage aliquots, which are thawed and used with limited further expansion. Continuous passage of STO fibroblasts without frequent screening should be avoided.

Plating an STO Feeder Layer Prior to Inactivation

Two different methods can be used to prepare an STO feeder layer: plate-then-irradiate and irradiate-then-plate. Most human EG cultures are derived using the former method. This is practical when a large g-irradiator is available. STO cells are passaged for short periods (not continuously) in PGC growth media without LIF, bFGF, or forskolin, and are disaggregated using 0.05% trypsin–EDTA solution. One day prior to use, a 96-well culture dish is coated with 0.1% gelatin for 30 minutes. The gelatin is withdrawn, and 5×10^4 STO cells are plated per well in PGC growth media without the LIF, bFGF, or forskolin. Similar cell densities ($\sim 1.5 \times 10^5$ cells/cm^2) can be achieved in other well configurations. The cells are grown overnight and then exposed to 5000rads (1rad = 0.01Gy) g-radiation or X-ray. The cells are then returned to the tissue culture incubator until required. Prior to use, the growth media is removed, 0.1 ml PGC growth medium with added factors is added to each well (or half of the required well volume), and the dish is returned to the tissue culture incubator.

Plating an STO Feeder Layer after Inactivation

This method of STO cell preparation is used when a large g-radiation unit is not available, when large amounts of cells are required, or if better control of STO cell density is required. STO cells are grown as described previously, trypsinized, counted, and resuspended in PGC growth media without added factors. The cells are placed into one or more 50 ml conical tubes, and placed into the g-irradiator or X-ray device. Cells are exposed to 5000 rads g-radiation or X-ray. Following exposure, cells are adjusted to a convenient concentration with PGC growth media without added factors, counted, and plated into tissue culture dishes that have been previously coated with 0.1% gelatin for 30 minutes. Cells are allowed to adhere overnight; then the media is replaced with half the final volume of PGC growth media, including factors prior to use.

Primordial Germ Cell Growth Media Components

Human EG cells are derived and maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 15% fetal bovine serum (FBS, Hyclone), 0.1 mM nonessential amino acids (Gibco BRL), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), 100 μ/ml penicillin (Gibco BRL), 100 mg/ml streptomycin (Gibco BRL), 1000 μ/ml human recombinant LIF (hrLIF, Chemicon), 1–2 ng/ml human recombinant bFGF (hrbFGF, R&D systems), and 10 mM forskolin (Sigma) prepared in DMSO.

Evaluation of Embryonic Germ Cell Cultures

Of 150 human PGC cultures initiated, 142 (~95%) demonstrated morphological, biochemical, and/or immunocytochemical characteristics consistent with previously-characterized pluripotent stem cell lines. The easiest and most consistent method for evaluating EG cultures is to note the presence of tightly-compacted multicellular colonies resembling early-passage mouse ES and EG cell colonies (Figure 42-2), rather than the flattened and more loosely associated colonies seen in human EC and rhesus ES cells. Under the best circumstances, the number of colonies should double or triple over a one-week passage. The trend is usually to start with small colonies, and to end with larger colonies that result from incomplete colony disaggregation (see the earlier section Subsequent Passage of EG Cultures).

High levels of AP activity are associated with human EG cells. Under standard culture conditions, human EG colonies are >70–90% AP+. As colonies differentiate, one can observe a lower staining percentage and weaker staining, sometimes restricted to the periphery of the colony.

Human EG cells have been further characterized by a bank of five monoclonal antibodies: SSEA-1; SSEA-3; SSEA-4; TRA-1-60; and TRA-1-81. Colonies stain

strongly for four of the five antibodies. The antibody recognizing SSEA-3 antigen stains the cells inconsistently and weakly. As with the results of AP staining, the percentage of cells within a colony that stain positive is variable.

The histological profile of human EG cells (AP+, SSEA-1+, SSEA-3+, SSEA-4+, TRA-1-60+, and TRA-1-81+) differs from undifferentiated human EC and rhesus ES cells, which are SSEA-1−. The fact that differentiation of the human EC line NTERA2 leads to increased expression of SSEA-1 may suggest that this is indicative of differentiation in the human EG cultures. However, NTERA2 differentiation is accompanied by the loss of the other markers, which are not observed in these cultures.

Karyotypic analyses carried out at passage 8–10 (60–70 days in culture) indicated apparently normal human chromosomes at the 300-band level of resolution. Both XX and XY cultures have been derived. Other markers of pluripotency, such as mRNA expression of the human ortholog of mouse Oct3/4 and telomerase enzyme activity, have been examined. Results differ greatly, depending on the status of the EG colony, culture, or both. In general, relatively undifferentiated EG colonies are OCT4 mRNA+ as detected by RTPCR and have detectable levels of telomerase.

Alkaline Phosphatase and Immunocytochemical Staining

Cells are fixed for detection of AP activity in 66% acetone–3% formaldehyde and then stained with naphthol/FRV-alkaline AP substrate (Sigma). For immunocytochemistry, cells are fixed in 3% paraformaldehyde in DPBS. Cell-surface glycolipid- and glycoprotein-specific monoclonal antibodies are used at 1:15 to 1:50 dilution. MC480 (SSEA-1), MC631 (SSEA-3), and MC813-70 (SSEA-4) antibodies were supplied by the Developmental Studies Hybridoma Bank (University of Iowa). TRA-1-60 and TRA-1-81 were a gift from Dr Peter Andrews (University of Sheffield, UK). Antibodies are detected by using biotinylated antimouse secondary antibody, streptavidin-conjugated horseradish peroxidase, and AEC chromogen (BioGenex).

Embryoid Body Formation and Analysis

Embryoid bodies (Ebs) form spontaneously in human EG cultures. Although this represents a loss of pluripotent EG cells from the culture, EBs provide evidence for the pluripotent status of the culture, and provide cellular material for subsequent culture and experimentation (see the section EBD Cells). Initially, EBs provided the only direct evidence that human EG cultures were pluripotent, as all attempts to form teratomas in mice from human EG cells failed. To this day, there is no evidence of teratoma formation from human EG cells or their derivatives.

Embryoid Body Embedding and Immunohistochemistry

The constituent cells of EBs can be identified most reliably by embedding them in paraffin and staining sections with a bank of well-characterized antibodies. This process avoids the significant problem of antibody trapping that complicates analyses of large three-dimensional structures when direct staining is attempted. EBs are collected from cultures and placed into a small drop of molten 2% low melting point agarose (FMC), prepared in DPBS, and cooled to 42ºC. Solidified agarose-containing EBs are then fixed in 3% paraformaldehyde in DPBS and embedded in paraffin. Individual 6 mm sections are placed on microscope slides (ProbeOn Plus, Fisher Scientific). Routinely, immunohistochemical analysis is carried out by using a BioTek-Tech Mate 1000 automated stainer (Ventana-BioTek Solutions). Manual staining is also carried out using standard immunohistochemical methods; however, antigen retrieval is required for some of the antibodies employed. Cryosections of EBs generally produce less satisfactory cell morphology and have not been used extensively.

Antibodies used on paraffin sections include HHF35 (muscle specific actin, Dako), M 760 (desmin, Dako), CD34 (Immunotech), Z311, (S-100, Dako), sm311 (panneurofilament, Sternberger Monoclonals), A008 (a-1-fetoprotein), CKERAE1/AE3 (pancytokeratin, Boehringer Mannheim), OV-TL 12/30 (cytokeratin 7, Dako), and K_S20.8 (cyto-keratin 20, Dako). Primary antibodies are detected by using biotinylated antirabbit or antimouse secondary antibody, streptavidin-conjugated horseradish peroxidase, and DAB chromogen (Ventana-BioTek Solutions). Slides are counterstained with hematoxylin. Using these and other antibodies it can be demonstrated that, when human EG cells differentiate, they form EBs comprised of endodermal, ectodermal, and mesodermal derivatives.

EMBRYOID BODY-DERIVED CELLS

Although a compelling demonstration of the potential of human EG cells, the limited growth characteristics of differentiated cells within Ebs, and the difficulties associated with their isolation, make extensive experimental manipulation difficult and limit their use in future cellular transplantation therapies. At least two possibilities exist to explain the presence of the observed differentiated cell types. These cells could be generated directly from the pluripotent EG cell, or they could proceed through a series of precursor or progenitor cell types, prior to the acquisition of a mature phenotype. It seems unlikely that cells within EBs would bypass normal pathways of differentiation, so efforts have been made to isolate and expand these populations. The hypothesis was that progenitor–precursor cells would have desirable proliferation characteristics, and could be recognized by the expression of

molecules known to mark progenitor–precursor populations, as well as by the simultaneous expression of markers normally considered part of a mature cellular expression repertoire. Simultaneous expression of neuronal and glial markers by neural progenitors and the expression of a variety of lineage-affiliated transcription factors and cytokine receptors by multipotent hematopoietic progenitors provide some basis for this hypothesis. In this model of differentiation, multilineage gene expression by precursor or progenitor cells defines a ground state from which cell-extrinsic and -intrinsic signals work to continuously define a differentiated expression pattern and phenotype, resulting in the developmental plasticity observed after the differentiation of bone marrow and central nervous system stem cells.

The method used to isolate cell populations from EBs is conceptually similar to microbiological selective media experiments. EBs are disaggregated and plated into several different cellular growth environments. These environments consist of combinations of a growth media and a matrix. Although many combinations were evaluated, most EBD cell cultures have been derived from one of six environments, formed by combinations of two growth media and three plating surfaces. The growth media are an RPMI 1640 media supplemented with 15% FBS, and a low (5%) FBS media supplemented with bFGF, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), and vascular endothelial growth factor (VEGF). The plating surfaces are bovine type I collagen, human extracellular matrix extract, and tissue culture-treated plastic. These are not intended to be highly selective environments. Instead, they favor several basic themes: cells thriving in high serum and elevated glucose (10 mM) conditions versus cells proliferating in low glucose (5 mM) under the control of four mitogens. Surfaces included binding to type I collagen, a biomatrix often thought to favor undifferentiated proliferation versus human extracellular matrix, a more complex mixture of laminin, collagen, and fibronectin. The initial assay is to determine conditions that favor extensive cell proliferation, with the hypothesis that this condition will favor undifferentiated cell populations, or at least provide numerical disadvantage to the terminally-differentiated constituents of the EB.

Embryoid Body-derived Growth and Expression Characteristics

Cell populations capable of long-term and robust proliferation can be isolated in this way from human EG-derived EBs. Embryoid body-derived is the generic term used to describe cells derived in this way *and* capable of extensive further proliferation. In general, the type I collagen and human extracellular matrices combined with the low serum media, provide the most rapid and extensive cell proliferation. EBD cell lines and cultures are routinely maintained in the environment in which they were derived. The EBD naming convention aids this process. The first two letters of the name refer to the EG culture from which it was derived. The second letter indicates the growth media (E for EGM2MV and R for RPMI1640), and the last letter indicates the matrix (C for collagen, E for human extracellular matrix extract, and P for plastic). For example, the EBD culture SDEC was derived from EG culture SD in EGM2MV medium on type I collagen.

To distinguish EBD cells from a simple population of cells, rapidly proliferating but presumably uninteresting in terms of molecules expressed, it is important to establish a robust expression profile assay. The assay should use redundant measures when possible and must combine breadth, sensitivity, specificity, and speed. A series of 24 RTPCRs detecting products from five cell lineages (neuronal, glial, muscle, hematopoietic–vascular endothelia, and endoderm) combined with immunocytochemical staining provides a rapid measure of cell expression. Inevitably, molecular markers are not as definitively specific as desired, so multiple markers for each lineage are advisable.

Using mRNA and antibody expression profiling, we can demonstrate that most rapidly proliferating EBD cell cultures simultaneously express a wide array of mRNA and protein markers normally associated with distinct developmental lineages. This is not a surprising property considering that EBD cells are, at least during the derivation stage, a mixed-cell population. More remarkable is the finding that most (11 of 13) EBD cell lines isolated by dilution cloning also exhibit a broad multi-lineage gene expression profile. It can also be demonstrated that the expression profile for a given EBD culture remains stable throughout the lifespan of the culture. This normally exceeds 70 population doublings, but is not unlimited since EBD cells are not immortal.

More than 100 EBD cell cultures and clonally isolated cell lines have been derived and characterized as described here. Most of these cultures share the properties of rapid and robust proliferation, and broad multi-lineage gene expression. Less than 10% of the EBD cultures derived have a narrow expression profile, with one extreme case expressing only nestin, vimentin, and a-1-fetoprotein mRNA. Other general trends in EBD expression are that many cultures appear to be neurally biased, with strong expression of neuronal, glial, and neural progenitor markers, and relatively weak in expression of muscle markers.

Other general characteristics of EBD cells are the relative ease with which they can be genetically manipulated, using lipofection and electroporation, as well as retroviral, adenoviral, and lentiviral vectors. Adenoviral and lentiviral vectors are capable of nearly 100% transduction efficiency. These techniques have been used to generate EBD lines that constitutively and tissue-specifically express enhanced GFP and contain many different genetic selection vectors. EBD

cells can be immortalized by retroviral-based expression of the telomerase RNA subunit (pBABE). Interestingly, after several hundred population doublings, these lines often become genetically unstable, generating at least two rearrangements: [47,XX,-1,+ del (1)(q12), +i (1)(q10)] and [46,XX, del(4)(p14)]. In addition, these EBD lines tend to have narrow expression profiles.

The imprinting pattern of several EBD cultures has been examined. In one study, expression levels of four imprinted genes (TSSC5, H19, SNRPN, and IGF2) were determined in five EBD cultures. Three of these genes (TSSC5, H19, and SNRPN) had normal monoallelic expression levels, and IGF2 had a partially-relaxed imprinting pattern comparable to levels found in normal somatic cells. This study also determined that the imprinting control region that regulates H19 and IGF2 imprinting had a normal pattern of DNA methylation. A second study determined that two XX EBD cultures had a normal pattern of X-inactivation.

The proliferation and expression characteristics of EBD cells suggest that they may be useful in the study of human cell differentiation, and as a resource for cellular transplantation therapies. One important property in this regard is that no tumor of human origin has arisen in any animal receiving EBD cells, although hundreds of mice, rats, and African green monkeys have received EBD transplants in a variety of anatomical locations, often consisting of more than one million cells injected. This is in contrast to the infrequent, yet significant, number of teratocarcinomas that have arisen following transplantation of cells produced through neural and hematopoietic differentiation of mouse ES cells.

Embryoid Body-derived Cell Transplantation

Transplantation of EBD cells into animal models of human disease constitutes an active and promising research avenue. Studies with EBD cells and many other cell types have suggested that tissue injury can be highly instructive to transplanted cells. This provides a powerful method to test the potential of cells to differentiate, without an initial understanding of the underlying mechanisms. These studies also suggest possibilities for the eventual treatment of patients suffering from these diseases.

One example of EBD cell transplantation is the use of an EBD culture named SDEC. This culture was initially selected for further study because of its strong neural expression bias. SDEC cells were introduced into the cerebrospinal fluid of normal rats and rats exposed to the neuroadapted Sindbis virus. This virus specifically targets spinal cord motor neurons, and infection results in permanent hind limb paralysis. SDEC cells transplanted into virally injured rats engrafted extensively the length of spinal cord and migrated into the cord parenchyma. Substantial engraftment was not observed in uninjured animals receiving SDEC cells. Engrafted SDEC cells took on expression characteristics of mature neurons and astrocytes. Remarkably, albeit at low frequency, engrafted SDEC cells became immunoreactive to choline acetyltransferase, and sent axons into the sciatic nerve. Even more remarkably, after 12 and 24 weeks, paralyzed animals receiving SDEC cells partially recovered hind limb function. In this experiment, the frequency and total number of neurons generated from SDEC cells was not sufficient to easily explain the significant recovery of function, which is not surprising since the transplanted SDEC cells are a mixed-cell population rather than a line or culture grown or differentiated to promote neural outcome. The mechanism proposed to explain the functional recovery involves EBD cells protecting host motor neurons from death, and facilitating host motor neuron reafferentation, possibly through the secretion of transforming growth factor-a and brain-derived neurotrophic factor.

This example illustrates several important points. The engraftment (and possibly one or more steps in cellular differentiation) of SDEC cells was promoted by an injury signal following viral infection. Once engrafted, cells within the SDEC population were capable of differentiation *in vivo* into mature astrocytes and neurons, some of which sent processes along the correct pathway to the sciatic nerve and were then capable of retrograde transport. It is difficult to see how this elaborate and spatially precise differentiation could be carried out *ex vivo* and then introduced into an animal or patient. The use of a mixed-cell population may have allowed the variety of cellular responses that ultimately resulted in functional recovery. Some cells within the population were capable of forming new neural cells, and others took on supportive and protective roles. Although, in this experimental model, the multiple roles carried out by use of a mixed-cell population may have resulted in functional recovery, future experiments will need to focus on isolation of sub-populations to increase the efficiency of differentiation into required cell types, and to address issues of safety. Lastly, the rats in all treatment groups received immunosuppressive drugs to discourage rejection of the human EBD cells. In the near term, this will likely be a feature of all EBD-based cellular transplantation experiments and therapies.

Embryoid Body-derived Derivation, Growth, and Cryopreservation Methods

EBs formed in the presence of PGC growth media are harvested in groups of 10 or more and are dissociated by digestion in 1 mg/ml Collagenase/Dispase (Roche) for 30 minutes to one hour at 37ºC. Cells are then spun at 1000 rpm for five minutes and resuspended in various growth media–matrix environments. These include RPMI

growth media (RPMI 1640 (LTI), 15% FCS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 μ/ml penicillin, and 100 mg/ml streptomycin) and EGM2MV media (Clonetics) (5% FCS, hydrocortisone, hbFGF, hVEGF, R^3-IGF1, ascorbic acid, hEGF, heparin, gentamycin, and amphotericin B). Matrices are bovine collagen I (Collaborative Biomedical, 10 mg/cm^2), human extracellular matrix (Collaborative Biomedical, 5 mg/cm^2), and tissue culture plastic. EBD cells are cultured at 37ºC, 5% CO_2, 95% humidity, and are routinely passaged 1:10 to 1:40 using 0.025% trypsin–0.01% EDTA (Clonetics) for five minutes at 37ºC. Low serum cultures are treated with trypsin inhibitor (Clonetics) and then spun down and resuspended in growth media. EBD cells are cryopreserved in the presence of 50% FCS, 10% DMSO, in a controlled rate freezing vessel and stored in liquid nitrogen.

SUMMARY

Human EG cells can be derived from PGCs by using methods similar to those used to derive mouse EG cultures. Like mouse ES and EG cells, human EG cells require LIF for proliferation as undifferentiated stem cells. Unlike mouse EG cells, however, human EG cells do not readily lose their dependence on exogenous cytokines and factors supplied by the feeder layer, and they have a higher frequency of spontaneous differentiation into EBs. Although EBs are a loss to the pluripotent stem cell population, they are a source of cells expressing markers of mature cellular phenotypes, as well as their presumed progenitors and precursors. Cells that retain a high capacity for cell proliferation and express makers of multiple lineages can be isolated from Ebs, and can be used in a variety of *in vitro* and *in vivo* differentiation paradigms. The current challenges are to match individual EBD cultures to desired endpoints, and to enrich or purify populations of cells within EBD cultures to more specifically address biological requirements.

KEY WORDS

Embryoid body A multicellular structure comprised of multiple-cell types formed during the *in vitro* and *in vivo* differentiation of embryonic stem cells.

Embryonic germ cell A pluripotent stem cell derived from primordial germ cells.

Primordial germ cell A cell type that colonizes the developing gonad and turns into egg or sperm.

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Chapter 43

Growth Factors and the Serum-free Culture of Human Pluripotent Stem Cells

Alice Pébay and Martin F. Pera

INTRODUCTION

The development of human embryonic stem cells (hESC) in 1998 presaged by work on stem cell lines from human embryonal carcinoma (EC) and monkey ES cells, led to a great surge of interest in the biology and potential therapeutic applications of pluripotent stem cells. The early reports of derivation of both monkey and hESC used serum-containing medium in combination with mouse embryo feeder cell support to maintain stem cell renewal, methodology that did not differ very much from those used previously to establish and propagate mouse ES cells or human EC cells. However, several critical differences in growth properties of mouse and hESC soon emerged. The human cells display very low cloning efficiency when dissociated to single cells, and the addition of leukemia inhibitory factor (LIF) is without effect on the growth of human stem cells in the absence of a feeder cell layer. Both of these properties of human pluripotent stem cells were first noted in early efforts to characterize human EC cells, and the findings meant that techniques that had been developed for efficient propagation and manipulation of mouse ES cells would find limited application in the human system.

The original methods for cultivation of hESC had several drawbacks. The use of fetal calf serum (FCS) and mouse feeder cells potentially exposes the human cells to unknown pathogens, in particular viruses or prions, which might be transmitted between species. Both serum and feeder cell layers also contain many undefined components that may have profound and undesirable effects on hESC differentiation. The poor growth of hESC following dissociation to single cells means that cloning at low densities, key to many experimental manipulations, is problematic. Moreover, because single cells survive poorly, and large aggregates of cells usually differentiate, the resulting requirement to subculture the cells as clusters of an appropriate size can limit the ability to scale-up the culture while maintaining cells in an undifferentiated state.

Thus, the major challenges to the development of hESC culture systems are as follows:

- to eliminate the use of serum in the medium;
- to eliminate the use of feeder cells;
- to improve the cloning efficiency; and
- to enable scale-up of the cultures.

Since the first reports of the development of hESC, there have been several advances in our ability to grow these cells *in vitro*, but not all of these goals have been achieved to date. This chapter reviews the current status of development of culture systems for hESC.

MOUSE EMBRYONIC STEM CELLS

The growth requirements of pluripotent stem cells have been studied in most detail in the mouse. The original reports of mouse ES cell derivation used embryonic fibroblast feeder cells and serum-containing medium. A key advance was the discovery of LIF as a critical component for maintenance of the pluripotent state. LIF and other members of this cytokine family that act through engagement of their particular receptors and the gp130 receptor maintain mouse ES cells in the pluripotent state through activation of JAK/STAT signaling. Production of LIF is one of the factors that accounts for the requirement of feeder cells to support mouse ES cell growth. Although many workers maintain mouse ES cells on a feeder layer in the presence of LIF, there are certainly a number of examples

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of germline-competent mouse ES cell lines that can be maintained without a feeder cell layer in the presence of LIF. Notwithstanding the evidence that LIF is important for mouse ES cell renewal, it is important to recognize that mice deficient in the gp130 receptor can still undergo normal development through midgestation, that mouse ES cell lines lacking the LIF receptor can still form colonies on a feeder cell layer, and that there is evidence that factors other than LIF can maintain mouse and human EC cells in the pluripotent state. These factors remain to be identified, but the important message for hESC work is that extrinsic pathways independent of gp130 signaling may have a role in pluripotent stem cell maintenance. Subsequent work has implicated bone morphogenetic protein signaling in stem cell maintenance in the mouse. Very recently, it has been shown that maintenance of mouse ES cells may not require any positive input at all; rather, blockade of signaling pathways involved in stem cell differentiation promotes self-renewal. In other words, in this species, pluripotency is the ground state of the stem cell (Ying *et al*., 2008). This exciting concept has yet to be extended to human cells.

HUMAN EMBRYONAL CARCINOMA CELLS

Human EC cell lines have been isolated by a number of investigators from different pathologic subclasses of these human tumors using different methodologies. Thus, although the stem cells of these cultures have certain common characteristics, including marker expression, the lines do vary in their ability to differentiate and in their growth requirements *in vitro*. One general observation is that many human EC cells, like hESC, are difficult to passage as single cells at low density. Although some human EC cell lines do not require feeder cell support, other pluripotent cell lines show a strong feeder cell dependence that is not met by addition of LIF to the culture medium. There is a spectrum of growth requirements among human EC cell lines that ranges from cell lines displaying a feeder cell requirement similar to that of hESC, to those EC lines that may be grown at clonal density at high efficiency in the absence of a feeder cell layer. In general, the more robust the human EC cell line in minimalist culture conditions, the more limited its capacity for differentiation.

Early efforts to analyze the growth requirements of human EC cells led to identification of several factors critical for their growth. One important attachment factor identified was the serum protein vitronectin, which on its own was able to support adhesion and subsequent growth of feeder-independent EC cell lines in the presence of insulin, transferrin, and albumin. Studies on feeder-cell-independent clones of the cell line Tera-2 revealed a key role for insulin-like growth factors in maintaining survival of pluripotent stem cells in the absence of serum. In addition to insulin-like growth factors, which are antiapoptotic for human EC cells, several factors active through tyrosine kinase surface receptors were identified with effect on human EC cells. Several groups reported a mitogenic effect of fibroblast growth factor-2 (FGF-2) on clones of the Tera-2 cell line grown under serum-free conditions; FGF-1 and FGF-4 were also active. At high doses, FGF-2 stimulated cell motility. Further studies pointed to a potential role for platelet-derived growth factor (PDGF) signaling in promoting growth of pluripotent human EC cells. The presence of receptors for this molecule was surprising because, in the mouse, expression of PDGF receptors occurs only after commitment of cells to mesoderm differentiation. There was indirect evidence for a potential autocrine role of this molecule in driving EC cell proliferation.

These observations on feeder-independent human EC lines can be contrasted to work with pluripotent, feeder-dependent human EC cell lines. These cells cannot be maintained without a feeder cell layer, even in the presence of serum; insulin, PDGF, FGF-2, and LIF, along with many other known factors, fail to replace the requirement of a feeder layer for stem cell maintenance, at least in the presence of serum.

Several candidate autocrine growth factor regulators were identified through studies of human EC cells. TDGF-1, also known as cripto, is an epidermal growth factor Cripto/FRL-1/Cryptic (EGF-CFC) superfamily member that is expressed in human EC cells and a few other cell types. Transcripts for a smaller, truncated form of this growth factor are more widely expressed in transformed cells and in normal human tissues. In a clone of the cell line Tera-2, recombinant TDGF-1 was found to be mitogenic. TDGF-1 is now known to be a co-receptor for Nodal. The transforming growth factor beta (TGFβ) superfamily member GDF-3 (also known as Vgr-1) is expressed in mouse and human EC cells and mouse ES cells. However, this member of the TGFβ superfamily is unusual, because it lacks a key cysteine residue involved in dimerization of most superfamily members, and because no biologic activity of GDF-3 either *in vitro* or *in vivo* has been described thus far. It has recently been identified as a BMP antagonist, and as a Nodal agonist. Finally, human EC cell lines and ES cell lines express receptors for the Wnt family of secreted regulators, and recent evidence implicates these molecules in the control of differentiation of mouse ES cells.

HUMAN EMBRYONIC STEM CELLS

Critical Components of the Original Culture Systems

Unlike mouse ES cells, hESC do not respond to LIF. Nonetheless, the first successful derivations of hESC used LIF in combination with a feeder layer of early passage

mouse embryonic fibroblasts in a medium optimized for the propagation of mouse ES cells, supplemented with serum (80% DMEM (no pyruvate, high glucose formulation), 20% fetal calf serum, 1 mM glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids). There have been few reports of the use of alternative basal media, and only a few groups have reported systematic studies of the role of low molecular weight components of the basal media in the support of hESC growth. Optimization of the basal medium can markedly affect the growth factor requirements of particular cell types *in vitro*, and more work in this area is certainly merited. In the original cell culture system used to establish hESC, omission of either feeder cells or serum leads to rapid differentiation of the hESC. Individual lots of serum or feeder cells can vary significantly in their ability to promote hESC growth. Moreover, the extent and range of hESC differentiation *in vitro* is also strongly influenced by the batch of serum or feeder cells used for growth.

Several groups have now described the growth of hESC on various types of human feeder cells, including fibroblasts, marrow stromal cells, and other cell types, and a recent report described derivation of hESC lines on STO cells, a permanent line of mouse embryo fibroblasts. Both human feeder cell layers and STO cells are much easier to maintain than primary mouse embryo fibroblasts, and the properties of the hESC grown on these cells appear similar to those grown on early passage embryo fibroblasts.

Elimination of the use of a feeder cell layer will require some understanding of the molecular basis of the support of stem cell renewal. In an effort to identify proteins produced by feeder cells that might be responsible for stem cell maintenance, proteomic analysis of secreted products found in conditioned medium of STO feeder cells have been carried out. One secreted protein identified of interest was the insulin-like growth factor binding protein (IGFBP)-4, which may play a role in presenting insulin-like growth factors to the cell. Other factors found in the STO-conditioned medium included pigment epithelium derived factor; a range of extracellular matrix components including SPARC, nidogen, and collagen alpha 1 and 2 chains; and several antioxidant proteins. This proteomic analysis probably was most likely to detect relatively abundant components of conditioned medium, and may well have missed critical membrane-bound regulators of stem cell growth and differentiation. One example of this latter class of protein is the BMP antagonist gremlin, a protein produced by fibroblasts that is mainly cell associated; gremlin transcripts are found in mouse embryonic fibroblast feeder cells used to support hESC growth, and BMPs, which are produced by hESC, have a profound differentiation effect on hESC. It is likely, although unproven, that feeder cell layers have multiple roles in the support of hESC growth, and that multiple proteins (cytokines, survival factors, antidifferentiative proteins, and extracellular matrix components) in either secreted or membrane-associated forms are involved in the interactions between feeder cells and stem cells.

Fibroblast Growth Factor-2, Insulin-like Growth Factor, and Heregulin

In recent years, hESC culture conditions have evolved from poorly-defined serum-containing medium to serum-free and, more recently, to chemically-defined medium. Fibroblast growth factor (FGF)-2 was the first factor found to be critical for hESC maintenance (Amit *et al.*, 2000), and many recently devised chemically defined systems for hESC growth incorporate this growth factor into their formulations. Human ESCs express receptors for FGFs, and also synthesize FGF-2. FGF-2 activates ERK signaling in hESC. Inhibition of this signal transduction pathway results in stem cell differentiation. FGF-2 was a key component of the first serum-free medium for the propagation of hESC. Basal medium (80% DMEM (no pyruvate, high glucose formulation), 1 mM glutamine, 0. 1 mM β-mercaptoethanol, and 1% nonessential amino acids) was supplemented with either 20% fetal calf serum, or 20% Knockout Serum Replacer, with or without 4 ng/ml FGF-2. Knockout Serum Replacer is a proprietary serum replacement consisting of amino acids, vitamins, transferrin or substitutes, insulin or insulin substitutes, trace elements, collagen precursors, and albumin preloaded with lipids. The composition of this serum replacement is not precisely specified by the manufacturer, and the description provides for the use of albumin substitutes, including other sources of animal protein such as embryo extracts. In this study, the cells were passaged every week. As noted previously, FGF-2 and other members of this growth factor family were shown to be mitogenic for human EC cells under serum-free conditions, but in the EC cell studies there was no indication that these factors inhibited differentiation. Since its first description, this serum-free method of hESC culture is now widely used, and allows for propagation of hESC on a larger scale than was previously possible using serum conditions.

However, the cloning efficiency of hESC in these conditions remains low, and feeder cells are still required. A further modification of this system replaced the feeder cell layer with a combination of conditioned medium from mouse embryonic fibroblasts and Matrigel, a commercially available basement membrane extract. This system also enabled long-term maintenance of hESC. However, both this system and the feeder cell-based culture method using FGF-2 supplemented media still incorporate an undefined mixture of animal products.

Although the effect of FGF on hESC is well-established, one study has indicated that the effect of FGF-2 on the stem cells is in fact indirect (Bendall *et al.*, 2007).

This work showed that, under certain conditions, ES cells spontaneously differentiate into a fibroblast-like cell that responded to FGF-2 and secreted IGF-2 into the medium. IGF-2 was found to affect ES cell survival and maintenance directly.

A recent study detected the expression of Erbb2 on hESC (Wang *et al.*, 2007). The ligand heregulin was therefore incorporated into a defined medium that supports ES cell growth for prolonged periods *in vitro*. The role of Erbb2 and its cognate signal transduction pathway in ES cell survival, proliferation, and maintenance remains poorly understood, but this additive is a key component of a proprietary defined medium for human ES cell growth.

Sphingosine-1-phosphate (S1P) and Platelet-derived Growth Factor (PDGF)

Chemically defined media (devoid of Knockout Serum Replacer) for hESC culture have been described, although few have demonstrated long-term maintenance of genetically stable hESC lines. Our laboratory analyzed components of serum most likely to be critical to hESC growth, and established the first chemically defined culture medium for the *in vitro* maintenance of hESC, by replacing serum from the culture medium by S1P and PDGF, two bioactive molecules present in serum (Pébay *et al.*, 2005). When added to serum-free medium in the presence of mouse embryonic fibroblasts, S1P and PDGF are able to maintain the hESC in the undifferentiated state for >80 passages. The serum-free culture medium used in this technique consists of DMEM (without sodium pyruvate, high glucose formulation) supplemented with 1% insulin/transferrin/selenium (ITS), 0.1 mM, β-mercaptoethanol, 1% nonessential amino acids, 2 mM glutamine, 25 mM Hepes, penicillin (50 U/ml), streptomycin (50 mg/ml), 10 μM S1P, and 20 ng/ml PDGF-AB. Cells were passaged every seven days. The cells remain diploid, retain markers of hESC, and are pluripotent as evidenced by differentiation *in vitro* and by formation of teratomas when grafted into immunodeprived animals. Short-term maintenance of hESC on Matrigel was also observed with this culture system. Although this technique presents the advantage of using a totally defined serum-free medium, a feeder cell layer is still required for optimal maintenance and clonal growth is very poor.

Transforming Growth Factorβ Superfamily: Activin/Nodal/TGFβ1

Much evidence now points to a role of the TGFβ superfamily in governing hESC self-renewal, and Activin/Nodal/TGFβ1 have now emerged as strong candidates for feeder-secreted maintenance factors for hESC (Vallier *et al.*, 2005). Nodal plays an important role in many processes in early embryonic development. Activin is often used as a surrogate factor for Nodal in cell culture experiments, because of its wider availability. Activin and Nodal have been shown to inhibit differentiation of hES cells. hES cells express receptors for Nodal (Activin receptor 1b, Activin receptor 2b, and Cripto or TDGF, a Nodal co-receptor). Various studies have shown that Activin/Nodal can synergise with FGF-2 or WNT to promote stem cell maintenance.

mTeSR1

Combining some of the factor requirements outlined above, Ludwig *et al.* (2006) successfully propagated hESC on various matrices in a novel chemically defined medium, mTeSR1 supplemented with FGF-2, TGF-β1, and other factors, in particular the GABAergic agonist pipecolic acid. This formulation is now available as a proprietary defined medium.

EXTRACELLULAR MATRIX COMPONENTS

The use of defined extracellular matrix components for the propagation of hESC is still under development. Most studies to date have relied on Matrigel. Ludwig *et al.* used a combination of extracellular matrix proteins from a variety of natural sources to support growth of ES cells in mTeSR medium. Recently, Braam *et al*. showed that recombinant vitronectin alone could provide for stem cell maintenance in defined medium, via engagement of the alpha v beta 5 integrin expressed on ES cells (Braam *et al.*, 2008).

FUTURE PROSPECTS

To date, existing hESC have been successfully sub-cultured in serum-free conditions, and new cell lines have been isolated using serum-free media containing FGF-2. With this shift to serum-free culture a movement has followed away from labor-intensive mechanical transfer of hESC colonies, and towards more straightforward enzymatic/low-calcium buffer methods of passage. However, concerns have been raised about the genetic stability of hESC cultured for prolonged periods of time under serum-free conditions using these passage techniques. Although these developments represent important steps toward the development of robust culture methodologies for propagation of hESC and eventual therapeutic use, some challenges remain, such as obtaining clonal growth at high efficiency. This step might soon be overcome, as new evidence suggests that the inhibition of the Rho/ROCK pathway increases dissociated hESC survival (Watanabe *et al.*, 2007).

Another challenge will be in the identification of the factors synthesized and released by the feeder cells, and by the stem cells themselves, and the identification of molecules produced by cells at early stages of differentiation that might influence stem cell fate. Furthermore, much effort has been made in defining new feeder-free and serum-free culture conditions for hESC, and different protocols are now available to maintain hESC undifferentiated. However, the signaling pathways involved in hESC self-renewal are still poorly understood. It is indeed likely that a combination of growth factors, through the sequential activation of multiple signaling pathways, is needed to maintain hESC self-renewal, survival, and pluripotency. Some signaling pathways probably enhance hESC cell survival and/or proliferation, while others may contribute to the maintenance of hESC in an undifferentiated state. To date, ligands from the TGFβ family (such as TGFβ, activin, and nodal), FGF, neurotrophins (BDNF, NT3, and NT4), S1P, and PDGF, and heregulin, have been identified as modulating hESC maintenance, while others such as bone morphogenetic proteins have been described as pro-differentiation factors in hESC. Understanding of the signaling pathways and their complex interactions that control hESC proliferation, survival, and pluripotency will prove to be an essential requirement for efficient manipulation of hESC towards human therapy.

KEY WORDS

Knockout Serum Replacer Proprietary serum replacement that consists of amino acids, vitamins, transferrin or substitutes, insulin or insulin substitutes, trace elements, collagen precursors, albumin preloaded with lipids.

Matrigel Commercially available basement membrane extract.

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Chapter 44

Feeder-free Culture

Holly Young, Tom Schulz, and Melissa K. Carpenter

INTRODUCTION

Because of their remarkable proliferative capacity and differentiation potential, human embryonic stem cells (hESC) may provide a source of cells for cell therapies, drug screening, and functional genomics applications. Derivation of hESC lines has been accomplished by culturing cells from the inner cell mass of pre-implantation embryos on mouse or human embryonic feeder cells (Thomson et al., 1998; Reubinoff et al., 2000; Amit and Itskovitz-Eldor, 2002; Richards et al., 2002), and many others. They have typically been maintained in media containing serum or serum-replacement supplemented with basic fibroblast growth factor (bFGF). In these conditions, the cells retain an undifferentiated state, and show stability in long-term culture (Amit et al., 2000; Rosler et al., 2004). In addition, hESC maintained on or off feeders express integrins a6 and b1, which may form a laminin-specific receptor, suggesting that the cells may interact with matrix components (Xu et al., 2001). These findings indicate that hESC require both soluble factors and matrix proteins.

A number of groups have developed methods for maintaining hESC in feeder-free conditions. Initially, we demonstrated that hESC can be maintained on Matrigel or laminin-coated plates in serum-free medium conditioned by mouse embryonic fibroblast (MEF) feeders (Xu et al., 2001). Cells maintained using these feeder-free conditions retain a normal karyotype and a stable proliferation rate; express SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase, hTERT, and OCT-4; and have the capacity to differentiate into cell types representing the three germ layers, both *in vitro* and *in vivo* (Xu et al., 2001). Furthermore, the cells maintain these features even after long-term culture over one year (Rosler et al., 2004). Cells maintained in feeder-free conditions can also be induced to differentiate, using specific culture conditions, into neural progenitors and neurons (Carpenter et al., 2001), endothelial cells, cardiomyocytes (Xu et al., 2002a; Laflamme et al., 2007), trophoblast (Xu et al., 2002b), hepatocyte-like cells (Rambhatla et al., 2003; Hay et al., 2008), oligodendrocytes (Nistor et al., 2005), and hematopoietic progenitors (Chadwick et al., 2003). Subsequently, many groups have developed feeder-free methods, as shown in Table 44-1. Most recently, we have developed techniques which include defined media and Matrigel (Wang et al., 2007). The utilization of defined media allows for standardization of reagents, more consistent undifferentiated cultures, and therefore more consistent differentiation outcomes. In this chapter, we present detailed protocols for the maintenance of hESC in feeder-free conditions using conditioned media and defined media, and then briefly describe methods for the characterization of hESC.

FEEDER-FREE CULTURE USING CONDITIONED MEDIUM AND MATRIGEL

Preparation of Conditioned Medium (CM) from Mouse Embryonic Fibroblasts

Harvesting Mouse Embryonic Fibroblasts

(Modified from *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, by E. J. Robertson, 1987.)

1. Anesthetize a 13-day pregnant mouse (we use CF-1 mice) using isoflurane or halothane. When the mouse is anesthetized, perform a cervical dislocation.
2. Saturate the abdomen with 70% ethanol, and pull back the skin to expose the peritoneum. With sterile surgical tools, cut open the peritoneal wall to expose the uterine horns. Remove the uterine horns and place them in a 10 cm Petri dish. Wash three times in 10 mL sterile PBS without Ca^{2+}/Mg^{2+}.
3. Cut open each embryonic sac with scissors, and release the embryos into the dish.
4. Using two pairs of watchmaker's forceps, remove the placenta and membranes from each embryo and dissect out the viscera. Place the embryos in a new Petri dish and wash three times with 10 mL sterile PBS.

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TABLE 44-1. Feeder-free Culture Conditions for Human Embryonic Stem Cells

Conditions	Cell Lines	Length in Test Conditions	Evaluation of Cells	References
On Matrigel with conditioned medium (20% serum replacement); 4 ng/mL bFGF	H1, H7, H9, H14	⩾ 1 year	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype, growth rates	Xu *et al.*, 2001
On Matrigel with 20% serum replacement; 8 ng/mL bFGF	H1, H7, H9	⩾ 40 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Rosler *et al.*, 2004
On fibronectin with 15% serum replacement; TGFB, LIF, and bFGF	I-6, I-3, H9	50 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype, growth rates	Amit *et al.*, 2004
On Matrigel with GSK-3-specific inhibitor	H1, BG01, BG02	7 days	Morphology, OCT4, pluripotency (*in vitro*, *in vivo*)	Sato *et al.*, 2004
On Matrigel with 20% serum replacement; bFGF, noggin, or high concentration of bFGF	H1, H9, H14	≈ 16–27 passages	Morphology, OCT4, pluripotency (*in vitro*, *in vivo*), karyotype, growth rates	Xu *et al.*, 2005b
On Matrigel with 20% serum replacement; 40 ng/mL of bFGF	H7, H9	6–11 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype, growth rates	Xu *et al.*, 2005a
On Matrigel with 20% serum replacement; 36 ng/mL bFGF	H1, H9	30 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype, growth rates	Wang *et al.*, 2005
On Matrigel or laminin with X-VIVO 10; 80 ng/mL bFGF	H1	12 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype, growth rates	Li *et al.*, 2005
On laminin with 20% serum replacement; 50 ng/mL Act, 50 ng/mL KGF, 10 mM Nic	HSF6	20 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Beattie *et al.*, 2005
On Matrigel with 20% serum replacement; 4, 24, 40, 80, 100, 250 ng/mL bFGF	H1, H7, H9, H14	≈ 30 passages	Morphology, OCT4, surface markers, pluripotency (*in vivo*)	Levenstein *et al.*, 2006
On Matrigel or fibronectin with HESCO; bFGF, Insulin, Transferrin, Wnt3a, April, cholesterol, albumin	H9, BG01	8 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Lu *et al.*, 2006
On Matrigel with N2/B27 CDM; 20 ng/mL bFGF	H1, HSF6	17 passages N2-CDM, 27 passages N2/B27-CDM	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Yao *et al.*, 2006
On combination human collagen IV, fibronectin, vitronectin, and laminin with TeSR1; 70 ng/mL bFGF, LiCl, GABA, pipecolic acid, 10 ng/mL TGFB1	H1, H7, H9, H14 and newly derived WA15, WA16	Passages 11–16	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Ludwig *et al.*, 2006
On Matrigel with 20% serum replacement; 50 ng/mL ea.BDNF, NT3, NT4	H1, H9	Passages 15–20	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Pyle *et al.*, 2006
On Matrigel, fibronectin or human serum with DC-HAIF; 10 ng/mL HRG1B, 10 ng/mL Act, 200 ng/mL LR-IGF1, 8 ng/mL bFGF	BG01, BG02, BG03, CyT49	7–9 mths, BG02 25 passages	Morphology, OCT4, surface markers, pluripotency (*in vitro*, *in vivo*), karyotype	Wang *et al.*, 2007

5. Transfer two embryos to a 35 mm culture dish. With curved iris scissors or a sterile razor blade, finely mince the tissue.
6. Add 2 mL trypsin-EDTA to the minced tissue and incubate the tissue for 5–10 minutes at 37ºC.
7. Neutralize the trypsin-EDTA by adding 5 mL MEF medium (DMEM high glucose, 10% FBS, 1 × glutamax and 1 × penicillin/streptomycin (optional)), and transfer the contents to a 15 mL tube.
8. Dissociate cells by triturating. Allow larger clumps to settle to bottom of the tube, and remove and plate the supernatant in 15 mL of MEF medium. We plate approximately two embryos in each T50 flask. Rock the flask back and forth to ensure an even distribution of the cells, and place in the incubator.
9. Split at 1:2 when cells are 80–85% confluent. This is usually the next day, in our laboratory.
10. When cells reach 80–85% confluence at this passage (passage 1), they can be maintained by splitting 1:2 every other day (see section on Thawing and Maintaining Mouse Embryonic Fibroblasts) and used for feeders or can be frozen (see section on Cryopreserving Mouse Embryonic Fibroblasts).
11. As with any new cell in the laboratory, a representative sample should be tested for mycoplasma and sterility.

Cryopreserving Mouse Embryonic Fibroblasts

1. Aspirate the MEF medium from each flask of cells to be frozen.
2. Wash the cells once in PBS without Ca^{2+}/Mg^{2+} (2–3 mL/T75 and 5–10 mL/T150).
3. Add trypsin-EDTA to the cells (1.0 mL/T75 and 1.5–2.0 mL/T150), and rock the flask back and forth to evenly distribute the solution. Incubate for approximately five minutes at 37ºC.
4. Detach the cells from the flask by pipetting or tapping the flask against the heel of your palm.
5. Neutralize the trypsin-EDTA by adding MEF medium (5 mL/T75, 10 mL/T150).
6. Gently pipette the cell mixture to break up clumps of cells. If clumps remain, transfer the suspension into a 50 mL tube and allow the chunks to settle out.
7. Perform a cell count of the suspension to determine the number of freezing vials required. We usually freeze 10–20 million cells per mL in each vial.
8. Pellet the cells by centrifugation for five minutes at 300 × g.
9. Aspirate the supernatant and resuspend the pellet in 0.5 mL of MEF medium containing 20% FBS per vial—i.e., one half the final volume required for freezing.
10. Dropwise, add an equivalent volume (0.5 mL per vial) of cryopreservation medium (80% cell culture medium, 20% DMSO) and mix. The resulting DMSO concentration should be 10%.
11. Place 1 mL of cell mixture into each freezing vial.
12. Rapidly transfer the cells to a Nalgene freezing container (Fisher, Cat.No. 15-350-50) and place at −70ºC overnight. Transfer the cells to liquid nitrogen the next day for long-term storage. Alternatively, cells can be frozen using a controlled rate freezer, and transferred to liquid nitrogen at the completion of the freeze cycle.

Thawing and Maintaining Mouse Embryonic Fibroblasts

1. Thaw a cryovial by gently swirling it in a 37ºC water bath, until only a small ice-crystal pellet remains in the tube. To avoid potential contamination, be careful not to submerge the cryovial above the level of the cap. Spray the outside of the vial with 70% ethanol.
2. Remove the contents of the vial by gently pipetting the cell suspension up and down once, and transfer to a 15 mL conical tube.
3. Slowly add 10 mL warm MEF medium to the tube dropwise, to reduce osmotic shock.
4. Centrifuge at 300 × g for five minutes.
5. Remove the supernatant.
6. Resuspend the cell pellet in 10 mL (T75) or 20 mL (T150) of culture medium, and transfer to a flask to be held in a 37ºC incubator. The plating density will need to be determined for each lot of MEF, usually ~5×10^4 to 1.5×10^5 cells/cm^2 are used.
7. Replace the medium the day after thawing. The MEFs are expanded by splitting 1:2 when they become 80–85% confluent, which is approximately every other day. It is important to keep cells subconfluent, so that they will not "contact inhibit." In our laboratory, MEFs are only used through passage 5.

Irradiating and Plating Mouse Embryonic Fibroblasts

1. Coat flasks or plates with 0.5% gelatin (15 mL/T225, 10 mL/T150, 5 mL/T75, and 1 mL/well of six-well plates) and incubate at 37ºC. Use one hour to one day after coating, removing the gelatin solution immediately before use.
2. Prepare MEFs for irradiation by aspirating the medium and washing once in PBS without Ca^{2+}/Mg^{2+} (2–3 mL/T75 and 5–10 mL/T150).
3. Add trypsin–EDTA (1.0 mL/T75 and 1.5 mL/T150), and incubate at 37ºC until cells are rounded up (usually 3–10 minutes). To loosen cells, either tap the flask against the heel of your palm or pipette them off.
4. Add 9 mL of MEF medium for one T150, then transfer the cell suspension into a 15 mL conical tube, and gently triturate several times to dissociate the cells.

Perform a cell count using a hemacytometer to prepare for plating.
5. Irradiate the cell suspension at 40–80 Gy. This number is variable among fibroblast sources. The goal is to stop proliferation without damaging the cells.
6. Spin down cells at 300 × g for five minutes and discard the supernatant.
7. Resuspend the cells in 10–30 mL of MEF medium. Plate at 56 000 cells/cm^2 for cultures to be used for conditioned medium (CM). The final volumes should be 3 mL per well for a six-well plate, 10 mL/T75, 20 mL/T150, and 50 mL/T225.
8. When placing the plate or flask in the incubator, gently shake it left to right and back to front to obtain an even distribution of cells.
9. Irradiated MEFs can be used for CM production five hours to seven days after plating.

Preparing and Storing Conditioned Medium (CM)

For convenience, CM can be generated and stored prior to use.

1. Plate irradiated MEFs at 56 000 cells/cm^2 in MEF medium, as described previously.
2. To generate CM, replace the MEF medium with hESC medium (Knockout DMEM, 20% Knockout Serum Replacement, 1 × non essential amino acids, 1 × glutamax, 0.1 mM 2-mercaptoethanol, 1 × penicillin/streptomycin (optional)) 0.5 mL/cm^2, supplemented with 4 ng/mL bFGF, one day before use.
3. Collect medium after overnight incubation and add an additional 4 ng/mL bFGF.
4. Add fresh hESC medium containing 4 ng/mL bFGF to the feeders. The MEFs can be used for one week, with CM collection once every day.
5. Pool and filter CM. Store at −20ºC for up to five months.
6. Thaw CM at 37ºC before using. Store CM at 4ºC, and use within one week of thawing.

FEEDER-FREE CULTURE USING CONDITIONED MEDIUM AND MATRIGEL OR LAMININ

Preparation of Matrigel or Laminin-Coated Plates

1. Slowly thaw Matrigel or Laminin aliquots at 4ºC to avoid gelling.
2. Dilute the Matrigel aliquots 1:30 in cold knockout DMEM or the Laminin aliquots in PBS without Ca^{2+}/Mg^{2+} to a final concentration of 20 μg/mL.
3. Add 1 mL of Matrigel or Laminin solution per one well of a six-well plate to be used or 1.5 mL matrix per 60 mm plate.
4. Incubate the plates for at least one hour at room temperature, or overnight at 4ºC. The plates can be stored at 4ºC for one week.
5. Remove coating solution immediately before use. Laminin-coated plates should be washed once with knockout DMEM before introducing cells.

Passage of Human Embryonic Stem Cells in Conditioned Medium on Matrigel or Laminin

1. Aspirate the medium from hESC, and add 1 mL of 200 units/mL collagenase IV per well of a six-well plate or 60 mm plate.
2. Incubate cells for 5–10 minutes at 37ºC. Incubation time will vary among batches of collagenase; therefore, determine the appropriate incubation time by examining the colonies. Stop incubation when the edges of the colonies start to pull from the plate.
3. Aspirate the collagenase and add 2 mL of CM supplemented with bFGF at a final concentration of 4 ng/mL into each well or plate.
4. Gently scrape the cells with a cell scraper or a 5 mL pipette to collect most of the cells.
5. For better cell adhesion and colony formation upon passage, dissociate the cells into small clusters (50–500 cells) by gently pipetting.
6. Seed the cells into each Matrigel or laminin-coated culture dish. The final volume of medium should be 3 mL per well or 5 mL per 60 mm plate. In this system, the hESC are maintained at high density. At confluence (usually one week in culture) the cells will be 300 000–500 000 cells/cm^2. We find that the optimal split ratio is 1:3 to 1:6. Using these ratios, the seeding density is 50 000–150 000 cells/cm^2.
7. Cells are maintained in a humidified incubator in a 5% CO_2−95% air atmosphere at 37ºC. Be sure to gently shake the plate left to right and back to front, or swirl in a "figure of eight" motion to obtain even distribution of cells.
8. The day after seeding, undifferentiated cells are visible as small colonies. Single cells between the colonies will begin to differentiate. As the cells proliferate, the colonies will become large and compact, taking most of the surface area of the culture dish (Figure 44-1).
9. For maintenance of feeder-free cultures, feed the hESC with 3–4 mL CM supplemented with bFGF per well of a six-well plate daily, or 4–5 mL per 60 mm plate. We find that one feeding can be skipped at day 2 after splitting. NOTE: Pre-warm media in a 37ºC water bath. Do not heat the entire bottle of medium, as the knockout DMEM and knockout serum replacement do not tolerate repeated warming and cooling.

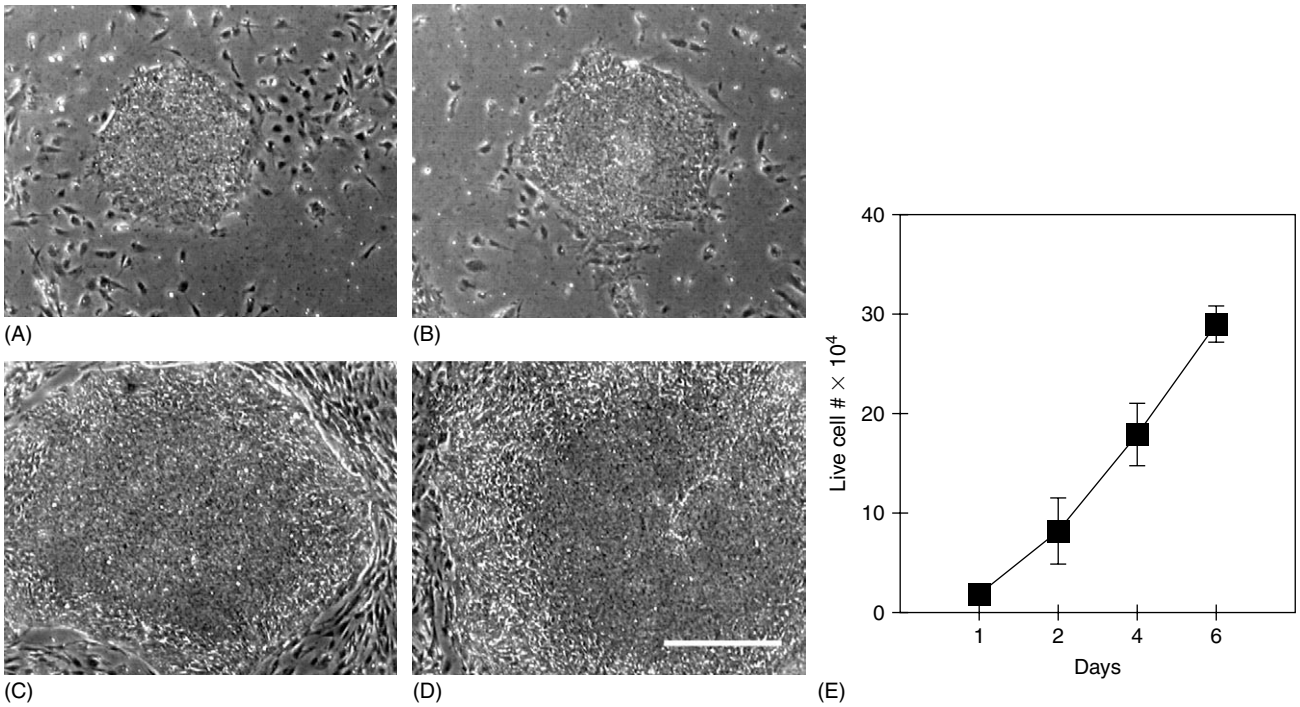


FIGURE 44-1 Growth of hESC in feeder-free conditions. Phase images of H7 (passage 31) undifferentiated hESC colonies maintained in feeder-free conditions were taken (A and B) two days; and (C and D) five days after seeding. Bar = 200 μm. (E) Proliferation of H7 cells (passage 24) was measured by counting live cells at various days after seeding. H7 hESC were dissociated with collagenase IV, resuspended in CM, and plated into Matrigel-coated 24-well plates. Cells were harvested with trypsin and counted at days 2, 4, and 6 after seeding. Each point on the graph represents the mean ± standard deviation of cell counts for three separate wells.

10. Passage when cells are 100% confluent, usually every 6–7 days. At this time, the undifferentiated cells should cover ~80% of the surface area, with the surrounding differentiated cells filling the remaining 20%. The cells between the colonies of undifferentiated cells appear to be stroma-like cells (Figure 44-1). The colonies (but not the stroma-like cells) show positive immunoreactivity for OCT-4 SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase.

FEEDER-FREE CULTURE USING DEFINED MEDIA AND MATRIGEL

A likely milieu of self-renewal and differentiation signals exists within hESC cultures. These arise from factors added to the growth media, factors secreted by feeders, the hESC, and by "spontaneously" differentiating cells, as well as cell–cell and hESC-matrix signaling interactions. The use of undefined components, such as partially purified albumin or ECM proteins, adds further complications because of variable carry-over of biologically active contaminants. An ideal culture system for hESC would contain: (1) a minimal set of components without conflicting self-renewal versus differentiation signals; and (2) only recombinant proteins; while being (3) inexpensive; (4) scalable; and (5) cGMP certified. In progressing toward these aims, several more advanced feeder-free and defined approaches have been reported (Table 44-1). Most were developed through iterative testing of different growth factors in backgrounds consisting of combinations of a rich basal media, serum replacement, the N2 and/or B27 supplements, or individual components from the above.

In order to build a defined medium based on an appreciation of the signaling pathways that are activated in hESC, we examined the phosphorylation status of receptor tyrosine kinases (Wang *et al.*, 2007). We determined that activation of the IGF1, Insulin, ERBB2, and ERBB3 receptors was a hallmark of proliferating hESC. IGF1R/IR activation could be attributed to microgram/mL concentrations of insulin in serum replacer and N2/B27 supplements, and provides a strong PI3 kinase/AKT signal (McLean *et al.*, 2007). Equivalent activation could be achieved with lower concentrations of LR^3-IGF1, an IGF1 analog. Phosphorylation of ERBB2/3 was consistent with the activity of an EGF family member, heregulin/neuregulin, in MEF-CM (Wang *et al.*, 2007). Inhibition of IGF1R and ERBB2 impacted self-renewal of hESC, as did small molecule inhibitors of Activin and FGF signaling (Vallier *et al.*, 2007; Robins and Schulz, 2009). Therefore, a simple defined media was developed, termed DC-HAIF, which consisted of DMEM/F12, non essential amino acids, trace elements, ascorbic acid, β-mercaptoethanol, penicillin/streptomycin (optional), with the only proteins

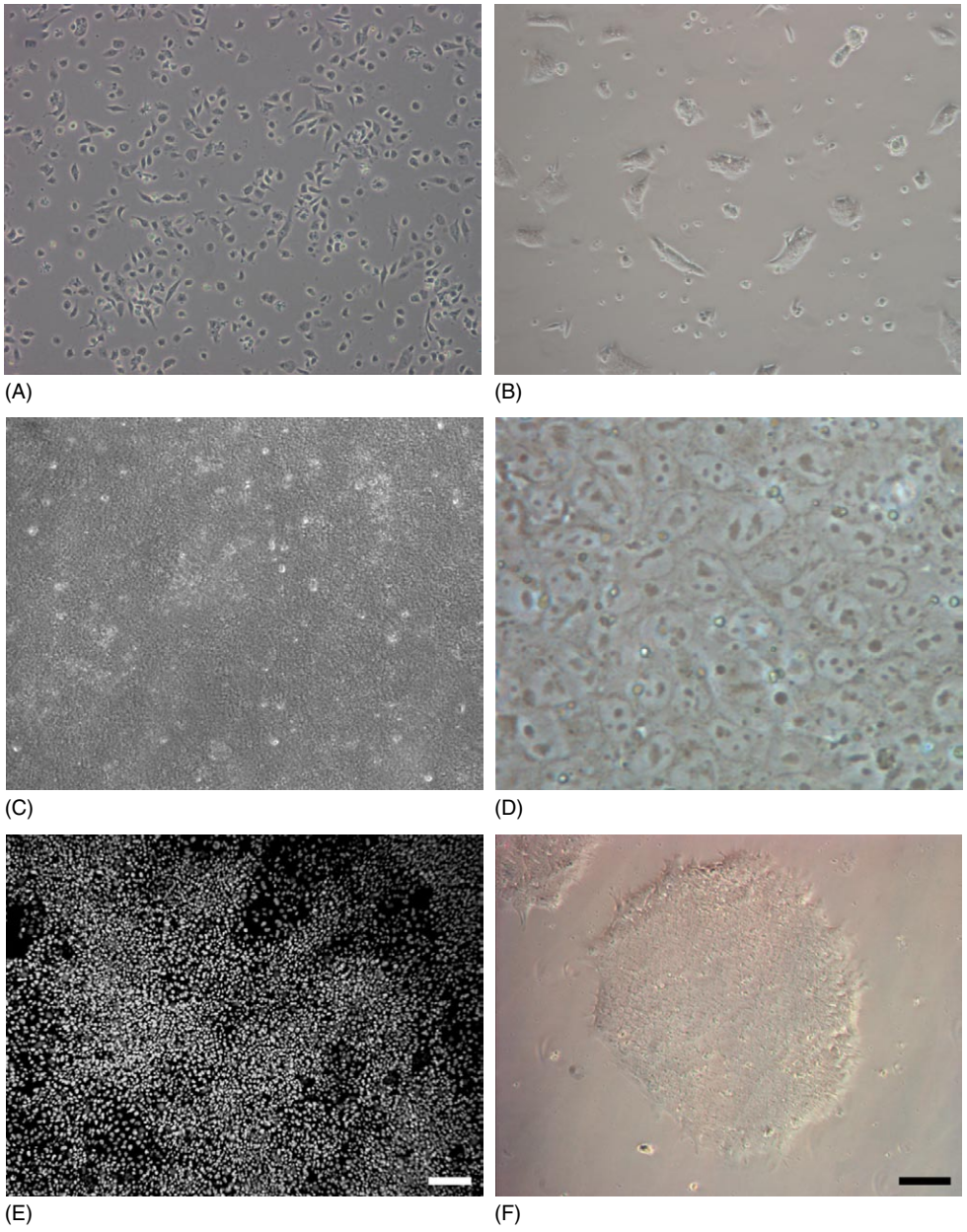


FIGURE 44-2 hESCs maintained in feeder-free conditions in defined medium. Accutase passaged BG02 cells in DC-HAIF medium at: (A) 3 hours; (B) 20 hours; and (C, D) 5 days post-plating. Essentially, single cell suspensions are generated at passaging, and typically >75% of cells survive, adhere, and migrate to form microcolonies within ~20 hours. Confluent monolayers of undifferentiated cells are generated as colonies expand and merge. (D) High magnification (40 ×) image of confluent BG02 cells in DC-HAIF. (E) OCT4 fluorescent immunostaining of a subconfluent BG02 DC-HAIF culture. (F) Collagenase passaged BG01 hESC growing in StemPro hESC SFM. Scale bars; A–C, F: 100 μm. E: 200 μm.

being caprylic acid-extracted fatty acid-free BSA, transferrin, and recombinant heregulin-1β, LR^3-IGF1 (an IGF1 analog), bFGF, and activin A. DC-HAIF supported long-term maintenance of pluripotent hESC, as well as single cell passaging and scaled expansion of hESC using Accutase (Figure 44-2), which is described in detail elsewhere (Wang *et al.*, 2007; Robins and Schulz, 2009). The methods below describe the culture of hESC in DC-HAIF on Matrigel using single cell passaging with Accutase. A batch-tested commercial formulation of DC-HAIF is also available from Invitrogen, termed StemPro hESC SFM. Coating with alternate humanized or more defined ECMs, such as fibronectin or human serum, can be used to substitute for the matrigel.

Passage of Human Embryonic Stem Cells in Defined Media with Accutase

The method lists volumes for standard serial propagation in 60 mm dishes. Prepare Matrigel-coated plate as

described in Preparation of Matrigel or Laminin-Coated Plates above, except use a 1:200 dilution.

1. Warm an appropriate amount of BSA wash medium (DMEM/F12, 0.2% BSA) and DC-HAIF medium (DMEM/F12, 2% BSA, 1 × each non-essential amino acids, trace elements and penicillin/streptomycin (optional), 50 μg/mL ascorbic acid, 10 μg/mL transferrin, and 0.1 mM β-mercaptoethanol) supplemented with 10 ng/mL HRG-β1, 10 ng/mL Act A, 200 ng/mL LR^3-IGF1, and 8 ng/mL bFGF, in a 37ºC water bath.
2. Aspirate the medium from the culture to be passaged, wash cells gently with DPBS without Ca^{2+}/Mg^{2+}, and aspirate.
3. Add 1 mL Accutase to cells, and rock the vessel gently to ensure all areas are covered. Leave three to six minutes and observe the disaggregation of colonies under a microscope.
4. Gently tap the sides of the vessel to dislodge cells, and remove with a pipette. Add to 5 mL BSA wash medium in a 15 mL centrifuge tube. Rinse the plate with fresh BSA wash medium, and add to tube.
5. Spin cells at 200 × g for four minutes at room temperature.
6. Aspirate the medium and gently flick the tube to loosen the cell pellet.
7. Gently resuspend the cells up to ~1 mL in DC-HAIF using a 5 mL pipette. Count viable cells using trypan blue exclusion.
8. For routine subculture, plate 10^6 cells per 60 mm growth factor reduced Matrigel coated dish (Matrigel at final concentration of 1:200), with a final volume of 4 mL DC-HAIF. When transitioning cultures from MEFs or MEF-CM cultures, plate 1.5 × 10^6 cells for the first 2–3 passages.
9. Place the vessels into an incubator set at 37ºC with 5% CO_2, and swirl in a "figure of eight" motion to disperse cells evenly.
10. Feed the cells daily with fresh DC-HAIF.
11. Passage by the above protocol every five to six days. Individual cells adhere, and then migrate, for the first ~20 hours, to form microcolonies. As the colonies expand and touch, the culture should form an undifferentiated and uniform confluent monolayer. Cultures are typically passaged one day after 100% confluence is reached, as continued proliferation occurs during this period. Typically 0.8–1.5 × 10^7 cells are generated per 60 mm dish.

Cryopreserving Human Embryonic Stem Cells

Pre-label the appropriate number of cryovials: typically 10^6 cells are frozen per vial.

Collect Cells Cultured in Conditioned Medium

1. Treat the cells with 200 U/mL collagenase IV for 5–10 minutes at 37ºC (until the edges of the colonies curl). Remove the collagenase and add 2 mL hESC medium per well.
2. With a 5 mL pipette, gently scrape colonies from the plate. Add the cell suspension to a 50 mL centrifuge tube, and gently break up the colonies. For cultures grown on Matrigel in CM, it is important to be gentle in this step, as larger clusters seem to result in a better yield on thawing. Ideally, colonies meant for freezing are left slightly larger than they would be for splitting. Proceed to step 3 in Collect Cells Cultured in Defined Medium below.

Collect Cells Cultured in Defined Medium

1. Collect a single cell suspension according to the Accutase passaging protocol above, and count the cells.
2. Centrifuge the cells for four minutes at 200 × g and aspirate the supernatant.
3. Resuspend the pellet in 250 μL of 50% cell culture medium, 50% FCS, per 10^6 cells—i.e., half the final volume required for freezing.
4. Dropwise, over two minutes with gentle swirling, add an equivalent volume (250 μL per vial) of cryopreservation medium (80% cell culture medium, 20% DMSO) and mix. NOTE: The final DMSO concentration is 10%.
5. Dispense 500 μL of cell mixture into pre-labeled cryovials and immediately transfer the cells to a Nalgene freezing container, place directly at −80ºC overnight. Transfer cells to liquid nitrogen the next day for long-term storage. Alternatively, cells can be frozen using a controlled-rate freezer, and transferred to liquid nitrogen at the end of the freeze cycle.

Thawing Human Embryonic Stem Cells

1. Thaw a cryovial by gently swirling it in a 37ºC water bath until only a small ice-crystal pellet remains in the tube, being careful not to submerge the cryovial. Spray the outside of the cryovial with 70% ethanol.
2. Remove the contents of the vial very gently and transfer the cells into a 15 mL conical centrifuge tube.
3. Slowly add 10 mL of warm CM, hESC medium, or DC-HAIF, dropwise (to reduce osmotic shock). While adding the medium, mix the cells in the tube by gently tapping the tube with a finger.
4. Centrifuge at 150 × g for five minutes, and resuspend in 0.5–1.0 mL of CM, or DC-HAIF.
5. Seed 0.5 mL per well of a four-well plate. For Accutase passaged cells in defined medium, seed 10^6 cells in 4 mL, in a 60 mm Matrigel-coated dish.

6. Change the medium daily. It may take two weeks before the cells are ready to be expanded. Passage defined medium cultures one day after 100% confluence is reached, which is typically 5–6 days after thawing.

Characterization of Feeder-free Human Embryonic Stem Cells

A collection of markers, including glycoproteins, glycolipids, and transcription factors, has been compiled to aid in the characterization of hESC. This panel consists largely of markers previously identified on human embryonic carcinoma (EC) cells, hematopoietic stem cells, and mouse ES cells (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Xu *et al.*, 2001; Amit and Itskovitz-Eldor, 2002; Henderson *et al.*, 2002; Richards *et al.*, 2002; Carpenter *et al.*, 2003; Hoffman and Carpenter, 2005). hESC maintained in feeder-free cultures have similar expression patterns to hESC maintained on feeders. Typical evaluation includes the assessment of: (1) the expression of undifferentiated cell markers such as OCT-4, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase; (2) telomerase activity; (3) karyotype; (4) proliferation rate; (5) *in vitro* differentiation capacity; and (6) teratoma formation.

Flow Cytometry Analysis of Combined SSEA-4 Surface Marker and OCT-4 Intranuclear Transcription Factor

1. When hESC cultures reach confluence, remove the culture medium and wash with DPBS without $Ca^{2+}Mg^{2+}$.
2. Dissociate and collect the cells for flow cytometry.
   a. Apply Accutase to the cell layer for three to five minutes, or until cells tap off.
   b. Collect cells in wash buffer (DPBS with 3% FBS) in a 15 mL conical tube.
   c. Centrifuge at ~300 × g for 7–10 minutes and aspirate supernatant.
3. Wash cells in surface staining buffer (DPBS, 0.1% BSA) (1 mL/10^6 cells) to remove residual protein, and transfer 10^6 cells to 75 mm tubes.
4. Surface staining: SSEA-4 AlexaFluor 488 (eBiosciences, 53-8843) or SSEA-4 PE (R&D Systems, FAB1435P), clone MC-813-70.
   a. Re-suspend cells in 50 μL SSEA-4 antibody at a 1/20 dilution in surface staining buffer.
   b. Incubate for 20 minutes at 4ºC.
5. Wash once in surface staining buffer to remove excess primary antibody.
6. Fix cell samples in 4% Paraformaldehyde/DPBS for 30 minutes in the dark at room temperature.
7. Wash cells in DPBS to remove fixative.
8. Permeabilize samples in DPBS with 0.2% Triton X-100 and 5% Normal Donkey Serum for 15–30 minutes at 4ºC. (NOTE: Cell pellets are not very tight and are easily disturbed.)
9. Wash in IC staining buffer (DPBS, 1% BSA) to remove detergent.
10. Intranuclear staining: goat anti-OCT-4 (SantaCruz, sc-8628).
    a. Re-suspend cell pellets in 50 μL OCT-4 antibody at 1/100 dilution in blocking buffer (DPBS, 5% normal donkey serum, 0.1% Triton X-100).
    b. Add 50 μL of buffer for unstained or secondary only controls.
    c. Cover samples securely with ParaFilm and incubate for one hour at room temperature or overnight at 4ºC.
11. Wash once in IC staining buffer to remove excess primary antibody.
12. Add secondary antibody, donkey anti-goat IgG-Cy5, (Jackson, 705-176-147).
    a. Re-suspend cells in 50 μL secondary antibody at a 1/8000 dilution in blocking buffer.
    b. Add 50 μL of buffer for unstained controls.
    c. Cover all tubes and incubate for 30–60 minutes at 4ºC.
13. Wash again to remove excess secondary antibody.
14. Resuspend cells in sufficient volume (~250–300 μL) for data acquisition by FACScalibur Flow Cytometer (Becton Dickinson, San Jose, CA) using CellQuest or FlowJo software.

Immunocytochemistry of Surface Markers

1. Passage hESC into Matrigel or laminin-coated chamber slides or coverslips and maintain the cells for 2–7 days.
2. Remove the medium and incubate the cells with primary antibodies—SSEA-4 (1:20), TRA-1-60 (1:40), and TRA-1-81 (1:80)—diluted in warm knockout DMEM at 37ºC for 30 minutes. In our laboratory, the antibody against SSEA-4 was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). TRA-1-60 and TRA-1-81 are available from Chemicon, MAB 4360 and MAB 4381.
3. Wash the cells with warm DMEM.
4. Fix the cells in 2% paraformaldehyde in DPBS for 15 minutes.
5. Wash the cells with DPBS (twice, five minutes each).
6. Block the cells by incubation in 5% normal goat serum (NGS) in DPBS at room temperature for 30 minutes.
7. Incubate with the fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1:125 in DPBS containing 1% NGS at room temperature for 30 minutes.

8. Wash the cells with DPBS (three times, five minutes each).
9. Mount slides with Vectashield mounting media (Vector Laboratories, Inc.) for fluorescence with 4′,6-diamidino-2-phenylindole (DAPI).

Detection of Alkaline Phosphatase

1. Passage hESC into Matrigel or laminin-coated chamber slides and maintain the cells for two to seven days.
2. Remove the culture medium, wash with DPBS, fix the cells with 4% paraformaldehyde for 15 minutes, and wash with DPBS.
3. Incubate the cells with an alkaline phosphatase substrate (Vector Laboratories, Inc., Burlingame, CA) at room temperature in the dark for one hour.
4. Rinse the slides gently once for two to five minutes in 100% ethanol before mounting.

Differentiation of Human Embryonic Stem Cells

Assessing the pluripotency potential of hESC is an essential part of characterizing. Extensive information is available with regards to differentiating hESC *in vitro* and can be found in chapters 42, 54–61.

In Vivo Differentiation: Formation of Teratomas

To date, teratomas have been generated from hESC by injecting the cells into immunocompromised mice. Injection of cells subcutaneously, intramuscularly, or under the kidney capsule will result in teratoma formation. The efficiency of tumor formation will be dependent on the cell line, the number of cells injected, the implant site, and the strain of the animal. In our laboratory, we generally use the following protocol.

1. When cells reach confluence, harvest the cells by incubating them in 200 units/mL of collagenase IV at 37ºC for 10 minutes. Be careful not to dissociate to single cells.
2. Wash the cells in DPBS, resuspend them at 1×10^8/mL, and store the cells in DPBS.
3. Inject the cells intramuscularly into severe-combined immunodeficiency, beige mice ($\sim 5 \times 10^6$ cells in 50 μL per site).
4. Monitor teratoma formation. After teratomas become palpable (five to eight weeks), they are excised and processed for histological analysis. Teratomas are benign tumors consisting of cell types representing all three germ layers.

MATERIALS FOR FEEDER-FREE HUMAN EMBRYONIC STEM CELL CULTURE

Reagents

Reagents for media culture of hESC, stock solutions, supplier and catalog number are provided in Table 44-2. Most solutions can be stored as aliquots at −20ºC but growth factors and CM should be held at −80ºC for long-term storage.

TABLE 44-2 Reagents for Feeder-free Human Embryonic Stem Cell Cultures

Reagent	Supplier	Catalog number
Media components		
Knockout DMEM	Invitrogen	10829-018
DMEM/F12, 1 ×	Invitrogen	10565-018
DMEM, 1 ×, high-glucose, without glutamine/sodium pyruvate	HyClone	SH30081.01
Knockout Serum Replacement	Invitrogen	10828-028
Fetal bovine serum	Hyclone	SH30071-03
Fatty acid-free BSA, 20% in DMEM/F12 NOTE: Store long-term at −80ºC. Requires batch testing. Do not substitute with lower grade.	Millipore	82-047-3
Trace Elements A, 1000 ×	Mediatech	99-182-C1

(*continued*)

TABLE 44-2 *(continued)*

Reagent	Supplier	Catalog number
Trace Elements B, 1000 ×	Mediatech	99-176-C1
Trace Elements C, 1000 ×	Mediatech	99-175-C1
Ascorbic Acid, 50 mg/mL in H_2O	Sigma	A4034
Bovine, 20 mg/mL or Human Transferrin, 4 mg/mL, in PBS	Invitrogen	11107-018 (b), 0030124SA (h)
Nonessential amino acids, 10 mM	Invitrogen	11140-050
GlutaMAX solution, 200 mM	Invitrogen	35050-061
Non-essential amino acids, 100 ×	Invitrogen	11140-050
Penicillin/streptomycin, 100 ×	Invitrogen	15070-063
2-mercaptoethanol, 1000 ×	Invitrogen	21985-023
StemPro hESC SFM, kit	Invitrogen	A1000701
Matrix components		
Growth factor-reduced or regular Matrigel, 1:1 in DMEM/F12	Becton Dickinson	35 6231 or 354234
Gelatin solution	Sigma	G1393
Human Laminin	Sigma	L-6274
Dissociation reagents		
Accutase, 0.5 mM EDTA	Innovative Cell Tech.	AT104
Collagenase IV solution	Invitrogen	17104-019
Trypsin–EDTA, 0.05% trypsin, 0.53 mM EDTA	Invitrogen	25300-054
Growth factors		
Recombinant Human Fibroblast Growth Factor, basic, 10 μg/mL in 0.1% BSA (PBS)	Invitrogen	13256-029
Recombinant bFGF, 25 μg/mL in 1% BSA(DMEM/F12)	Sigma	F0291
Recombinant Activin A, 25 μg/mL in 0.2% BSA(PBS)	R&D Systems	338-AC
Recombinant Human Heregulin-β1, EGF domain, 10 μg/mL in 0.1% BSA(PBS)	PeproTech Inc	100-03
Recombinant LR^3-IGF1, 1 mg/mL in 10 mM HCl	Sigma	I1271
Other reagents		
Dimethyl sulfoxide (DMSO)	Sigma	D2650
Embryo transfer water	Sigma	W1503
Sterile PBS without Ca^{2+}/Mg^{2+}	Invitrogen	14190-144

Tissue Culture Plates and Flasks

We use six-well plates (Falcon, Cat. No 3046) for hESC culture; a T75 flask (Corning, Cat. No. 430641), T150 flask (Corning, Cat. No. 430825), and T225 flask (Corning, Cat. No. 431082) for MEF culture; six-well low-attachment plates (Corning, Cat. No. 29443-030) for embryoid bodies (EBs); and four-well plates for thawing hESC (Nunc, Cat. No. 176740).

Incubators

All cells are maintained under sterile conditions in a humidified incubator in a 5% CO_2:95% air atmosphere at 37ºC.

KEY WORDS

Cryopreservation Freezing of a cell population or cell line in specialized media that allows storage of the cells for prolonged periods of time. Frozen cells can then be thawed and propagated at a later date.

Feeder-free Cells that are cultured without direct contact with feeder layers.

Feeder layer A layer of cells that are growth-arrested, providing nutrients, growth factors, and matrix components to a second cell population.

Inner cell mass (ICM) The cluster of pluripotent cells located at one pole of the inner surface of the blastocyst; these cells will form the body of the embryo after implantation.

Split/Passage Transfer or subculture of cells from one culture vessel to another, resulting in the expansion of the cell population.

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Chapter 45

Genetic Manipulation of Human Embryonic Stem Cells

Yoav Mayshar and Nissim Benvenisty

Genetic manipulation, the process of inducing changes in gene expression and the expression of novel genes, has proven to be an indispensable tool in recent genetic research. The implementation of increasingly powerful genetic tools to mouse embryonic stem (ES) cells has led to an explosion of data concerning the specific properties of an extremely large array of genes. Current available techniques allow for the specific elimination of target gene expression, tissue specific induction of reporter gene expression, the overexpression of cellular genes, and more. It is not surprising, therefore, that a short time after the derivation of human ES cells, several studies describing genetic manipulation of these cells have been reported. This chapter focuses on the recent advances in the genetic manipulation of human ES cells, the methods currently available, and their possible utilization. A brief discussion of advanced genetic manipulation techniques not yet demonstrated in these cells is also presented.

INTRODUCTION

The new technology of establishing human embryonic stem (ES) cell lines has raised great hopes for breaking new ground in basic and clinical research. Clearly, genetic manipulation will play a pivotal role in applying this technology to biological research, as well as for the specific needs of transplantation medicine.

Genetic manipulation has proved to be a key experimental procedure in the field of mouse ES cell research since their initial isolation, over 20 years ago. Many effective techniques have since been developed for bringing about specific genetic modifications. Some of these methodologies have recently been adapted for the manipulation of human ES cells. These include both transfection and infection protocols, as well as overexpression and homologous recombination procedures. The newly-acquired availability of these techniques should make genetic engineering of human ES cells routine, and enable great advances in the field of human ES cell research.

By introduction of genetic modifications to mouse ES cells, a great number of genetically modified animals have been produced, which have proved to be an extremely valuable tool for research. Obviously, no equivalent research can be conducted in humans. Thus, despite the great potential attributed to human ES cells, much of the basic developmental research using these cells may be limited to *in vitro* studies. Researchers into human ES cells are, therefore, forced to adopt developmental models such as embryoid bodies (EBs) for studying early embryogenesis. This chapter will present the principle aspects regarding the *in vitro* genetic manipulation of human ES cells, and the possible uses of the variety of methods currently available.

METHODS OF GENETIC MANIPULATION

A variety of methods are currently used for manipulating mammalian gene expression. Many of these have been used in mouse ES cell research. Of these, several gene delivery methods have been shown to be effective in human ES cells. These include transfection by various chemical reagents, electroporation, and viral infection. Transfection is probably the most commonly used method for genetic manipulation. The system is straightforward, and relatively easy to calibrate. It provides sufficient numbers of cells for clonal expansion, and allows for insertion of constructs of virtually unlimited size. Recently, long-term transgene expression in ES cells was demonstrated using infection by lentiviral vectors. This technique has been shown to be highly effective, and could therefore emerge as a popular tool in human ES cell genetic engineering.

Transfection

The method used for gene delivery is one of the major variables that directly affect the efficiency of genetic manipulation. The choice of the gene delivery method should be based, first and foremost, on the nature of experiment to

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be performed. The duration of the genetic modification is one factor that will dictate the method of choice. For short-term induction, transient expression can be achieved through the introduction of supercoiled plasmid DNA, transcription from which has been shown to be more efficient than from the linear form. Transcription from transiently introduced plasmids usually peaks at around 48 hours' post-transfection. For the most part, positive cells demonstrate relatively high expression levels, as a result of a high copy number of plasmids being introduced into transfected cells. Stable transfection is accomplished by selecting for cells where the vector DNA has integrated into the genome. In this type of experiment, it is important to linearize the vector, as this significantly raises integration and targeting efficiencies. A positive selection marker under regulation of a strong constitutive promoter is frequently included in the insert when the gene target is non-selectable. Selection should not be carried out immediately following transfection. Delaying the selection gives the cells time to recuperate and express the resistance marker. Given that the various selection reagents are toxic to all mammalian cells, the feeder layer supporting the cells must also be resistant to the selection drug. To this end, lines of transgenic mice, carrying various genes conferring resistance to selection drugs, are currently available for the derivation of resistant feeder cells.

Transfection Reagents

In the case of chemical-based transfection, carrier molecules bind the foreign nucleic acids, and introduce them into the cells through the plasma membrane. In general, the cellular uptake of the exogenous nucleic acids is thought to occur either through endocytosis, or in the case of lipid-based reagents, through fusion of a lipidic vesicle to the plasma membrane. This method can routinely produce transient transfection rates of approximately 10%, and stable transfection efficiency of 10^{-5}–10^{-6}. The main advantages of chemical transfection are, notably, the relative simplicity of the procedure, and the fact that it can be performed on an adherent cell culture.

Electroporation

Transfection by electroporation was adopted early on as the method of choice for gene-targeting in mouse ES cells. This process involves applying a brief high voltage shock to cells in suspension, in the presence of DNA molecules. The electric shock is thought to cause transient pores to open up in the cell membrane, enabling DNA to enter. A recent study performed by Zwaka and Thomson demonstrated the efficiency of electroporation for the production of transgenic HESC clones. Importantly, the mouse electroporation protocol that proved inadequate for HESCs was customized for these cells. Using electroporation, a stable integration rate of $\sim 10^{-5}$ was achieved and homologous recombination efficiencies were reported to be between 2–40%, subject to the properties of the vector employed. A substantial number of homologous recombination clones have thus been generated for a number of different vector constructs, demonstrating the feasibility of achieving efficient gene-targeting in human ES cells.

Infection

Unlike transfection methods, viral vectors can produce a very high percentage of infected cells. Viral vectors also allow easy determination of the vector copy number, both for transient and stable expression. Several groups have recently reported transgene expression in human ES cells using lentiviral vectors. Lentiviral infection provides a very high proportion of stable integrants, although integration cannot be targeted, and vector size may be an additional limiting factor. Because of its efficiency, this method could prove useful for bypassing the need for selection and time-consuming clonal expansion, as well as for experiments intended to induce random insertional mutagenesis or gene trap.

GENETIC MODIFICATION APPROACHES

One of the major roles of genetic engineering will be research into the early stages of human development, and the genes involved in this process. The roles of many of these genes have been elucidated using genetic manipulation techniques, mostly with the aid of genetically-modified animals. Several studies using EB models have shown that specific early embryonic developmental processes can, to some extent, be recapitulated *in vitro*. Human ES cells thus provide researchers with a unique system to investigate the early stages of embryonic development through the implementation of genetic manipulation techniques. These techniques can also be used to determine gene function at the cellular level.

Several approaches can be used to discover the activity of specific genes in ES cells through genetic manipulation. These include overexpression of cellular or foreign genes, use of reporter genes, and gene silencing. In this section we attempt to summarize the major techniques currently used in human ES cells, together with a number of other techniques that are likely to be implemented in human ES cell research in the near future (see Figure 45-1).

Exogenous Gene Expression

The concept of directed differentiation is an example of the possible utility of introducing exogenous properties into human ES cells. Extensive studies are currently being carried out on directed differentiation in human ES cells. The addition of specific growth factors to EBs during the differentiation process has been shown to modify their

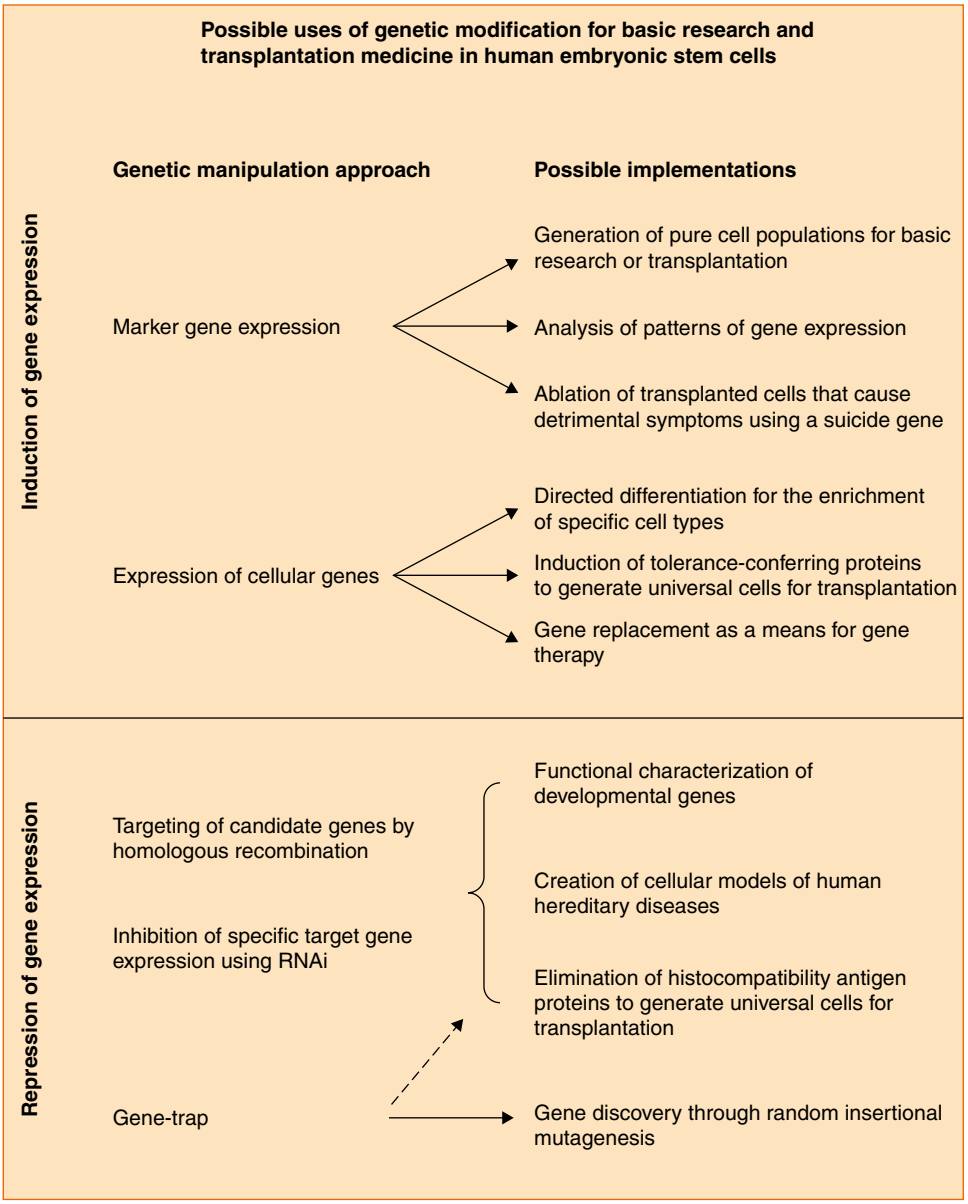


FIGURE 45.1

differentiation potential. Use of various enrichment protocols has made it possible to obtain several different cell types, such as neurons, hematopoietic cells, and cardiomyocytes. While creation of substantial amounts of certain cell types has been reported, the outcome of some differentiation protocols are often inconsistent, and are currently unable to yield the pure cell populations required for transplantation. Genetic manipulation could complement these methods, and facilitate the practical application of human ES cells in transplantation medicine.

Genetic Labeling

Introduction of constitutively expressed reporter genes, such as the gene encoding for green fluorescent protein (GFP) into the cells, allows their detection in a mixed population. This method was applied in a transplantation experiment where undifferentiated human ES cells were introduced into an early stage chick embryo. By using the same reporter gene regulated by a promoter of an ES cell-enriched gene (*Rex1*), it was possible to follow undifferentiated cell populations during differentiation *in vitro*, at the same time screening out differentiated cells with a fluorescence-activated cell sorter (FACS). Accordingly, Zwaka and Thomson created knockin cell lines using GFP or neomycin resistance promoterless constructs. Through this method, in the homologous recombination events that were selected, GFP expression was regulated by the endogenous OCT4 promoter. By placing the reporter under the regulation of the native gene, possible position effects that could confound the interpretation of the results are avoided, and the entire set of *cis*-acting elements is utilized.

Alternatively, the introduction of a conditional suicide gene could solve one of the principle concerns facing cellular transplantation. Adverse effects from transplants may occur as a result of malfunction or uncontrolled proliferation of grafted cells. Selective ablation of human ES cells expressing herpes simplex virus thymidine kinase (HSV-*tk*) has been demonstrated in an animal model. Growth of teratomas formed by transplanting PGK-HSV-*tk* transfected cells was successfully arrested by oral administration of the prodrug gancyclovir. Using this technique, it will be possible to transplant "fail-safe" cells into patients. These cells could then be eliminated on the onset of harmful symptoms.

Probably the most direct way for the enrichment of a specific cell type using genetic manipulation is to isolate it, using a tissue specific promoter driving a reporter or resistance gene. This method has been demonstrated for the enrichment of undifferentiated human ES cells, but can also be applied to isolate specific populations of differentiated cells. In mouse ES cells, through expression of a neomycin resistance gene regulated by the endogenous promoter of *sox2*, a neuronal progenitor specific gene, Li *et al.* (1998) produced highly-enriched neural precursors from a heterogeneous population following selection. Similarly, mouse cardiac cells were isolated by using a reporter gene driven by the cardiac alpha actin promoter. Of course, the efficiency of such direct selection methods could be improved under culture conditions that allow greater quantities of the desired cell type to be produced.

Bacterial Artificial Chromosomes and Recombineering

One of the major problems in reporter gene expression is to faithfully recapitulate the endogenous gene. Conventional DNA constructs are of limited size, and are therefore constrained by the maximal size of control elements that can be included. This can be overcome, as has been previously described, by a knockin approach—even though this is a difficult procedure and also disrupts one of the copies of the gene. Bacterial artificial chromosome (BAC)-based constructs containing cloned human DNA have been combined with homologous recombination in *E. coli* (recombineering) to facilitate in solving this problem. The basis of the method is the use of publicly and commercially available BACs containing cloned genomic regions of 100–200-kb, together spanning practically the entire human genome. Thus, it is possible to obtain a BAC containing virtually any gene of interest, along with extensive upstream and downstream sequences. These flanking sequences could be critically important for faithfully recapitulating gene expression by long-distance *cis* regulatory elements. Utilization of the BAC for genetic manipulation is facilitated by the use of recombineering. This procedure takes advantage of phage λ recombination proteins that induce homologous recombination within *E. coli*, using only very short homologous flanking sequences. Thus, BACs have been used to modify mouse ES cells, and transgenic animals have been established. In these experiments, reporter genes were the main use of the constructs, although targeting vectors for genomic modification were also demonstrated.

Over-expression of Cellular Genes as Dominant Effectors of Cell Fate

The overexpression of developmentally important genes is currently being used as a means of achieving directed differentiation. Several regulatory genes have been introduced in various mouse experiments. Hepatocyte nuclear factor 3 (HNF3) transcription factors -α and -β induced differentiation of mouse ES cells towards the early endodermal lineage. Ectopic expression of HoxB4, a homeotic selector gene, has enabled the use of mouse ES cells for the repopulation of myeloid and lymphoid lineages in immune-deficient mice. Similarly, the expression of the nuclear receptor related-1 (Nurr1) transcription factor generated a highly-enriched population of midbrain neural stem cells that have been shown to ameliorate the phenotype of a mouse model of Parkinson's disease.

As yet no single key factor or gene has been found that could cause undifferentiated ES cells to differentiate *en masse* to a specific lineage or cell type. Therefore, a combination of genetic manipulation, and selective culture conditions, would probably be necessary to generate differentiated transplant ready cells from undifferentiated ES cells.

Gene Silencing

As opposed to the exogenous expression of various genes, gene silencing assesses the functionality of a particular gene of interest through its elimination. In the mouse, gene silencing is commonly achieved through homologous recombination, in which an exogenous sequence is inserted within the gene's coding region, thus disrupting it. Gene trap is an additional method through which a wide range of genomic sequences can be randomly mutated. These techniques that generate knockout alleles are, however, of more limited use in human ES cells, as will be discussed later. Thus, in these cells the most common emerging method for gene silencing is post-transcriptional RNA interference—also known as knockdown.

Gene Targeting

Genetic modification has, for many years, been considered a cornerstone of mammalian biology research. Two major advances in genetic manipulation of the mammalian genome were the isolation of mouse ES cells, and the development of targeting vectors. Together, these two methods have enabled

the relatively straightforward production of genetically modified mice, carrying predesigned alterations to their genome. The subsequent proliferation of data has shed light on the functional characteristics of a very large number of genes, and has led to the creation of various models for human disease. Although a number of mouse models have been created, some have proved inadequate in recapitulating some or all aspects of the human phenotype. Research is often limited by lack of an adequate mouse model, and working with primary cultures can often be problematic. In light of these difficulties, the creation of a cellular model of human disease, using human ES cells, will be very valuable.

Mutation of the hypoxanthine guanine phosphoribosyltransferase gene (HPRT) in humans is the basis for Lesch–Nyhan syndrome, a severe genetic disease. This syndrome is manifested in a variety of problems related to high uric acid levels. These include gout-like arthritis and renal failure. There are also neurological disorders associated with this disease, but the exact causes are still unknown. Mouse models for HPRT deficiency failed to fully-recapitulate the human disease, possibly because uric acid metabolism is different in humans. Homologous recombination was recently used to disrupt HPRT using positive negative selection. Analysis of mutant human ES cell metabolism showed that, unlike their mouse counterparts, human cells mimic the Lesch–Nyhan phenotype.

HPRT is ideal for analyzing homologous recombination, as it is X-linked. Thus, mutation of the single allele in male cells may produce a phenotype. Furthermore, cells lacking HPRT activity can be selected with 6-thioguanine (6TG). By culturing these cells in HAT medium, they can be selectively eliminated. In many cases, the rate-limiting step towards the obtaining of targeted recombinants is the high number of nonhomologous integrations, making screening for homologous recombinants very time-consuming. The additional negative selection is useful, in order to confirm successful homologous recombination events and eliminate false positives. Thus, by using an HPRT– cell line as a base for further genetic manipulation, an HPRT minigene can be used as an additional positive and negative selection, by correcting the HPRT deficiency. These advantages have made HPRT very useful in the initial study of homologous recombination. In cases other than HPRT targeting, a vector is usually constructed that contains a positive selection cassette (usually the neomycin-resistance gene), flanked by sequences homologous to the endogenous target gene. A negative selection marker, usually HSV-*tk*, is often placed in such a way that, following linearization of the vector, it is located outside the region of homology. Thus, in those cases in which homologous recombination has successfully occurred, the transfected cells will not contain the HSV-*tk* gene, and will be resistant to the drug gancyclovir.

When targeting expressed genes, promoterless constructs can be employed, in order to increase the efficiency of homologous recombination by reducing the selectability of most random integrations. This method requires careful calibration of the concentration of the selection reagent, subject to the strength of endogenous promoter. Zwaka and Thomson (2003) targeted the gene encoding OCT4 (*POU5F1*) using this approach, and achieved homologous recombination rates of up to 40%.

As opposed to gene-targeting, random gene disruption on a wide scale is currently being performed by gene trap. In this method, a reporter gene lacking an essential regulatory element is randomly inserted into the genome. Thus, in cases in which the reporter gene is expressed, often this is due to the use of an endogenous regulatory sequence. A large-scale disruption of genes is therefore made possible, allowing for the discovery of new genes, and the creation of banks for a wide variety of mutations.

Induction of Homozygosity in Mutant Alleles

Several methods have been developed for the homozygotization of mutated alleles in mouse ES cells. When mutating the mouse genome, there is often an option of generating homozygosity through breeding two heterozygotes. However, research with human models will require *in vitro* manipulation. Two strategies can be employed to this end: (1) targeting the remaining allele by a second round of homologous recombination using distinct selection markers; and (2) growing transfected cells in high concentrations of the selection reagent, thus selecting for spontaneous homozygotization of the mutated allele containing the resistance gene.

The Effect of Extent and Nature of Homology on Targeting Efficiency

The length of the homologous regions has been shown to be a critical factor in determining the effectiveness of targeted recombination. By increasing the length of homology from 2-kb to 10-kb, an exponential increase in targeting efficiency is achieved. A less dramatic rate of increase is observed with up to ~14-kb of homology. The length of the short arm of homology should not be less than approximately 0.5-kb. Use of isogenic, rather than non-isogenic, DNA has also been shown to increase the rate of targeted recombination dramatically, although the need for isogenic DNA for human somatic cell lines and ES cells has recently been disputed.

Advanced Methods of Gene Targeting

It has been shown that the strategy of inserting a selection cassette to generate a knockout phenotype can confound the interpretation of these experiments, as the exogenous promoter may disrupt endogenous gene expression at

distances of over 100-kb from the point of insertion. This disruption of the local regulation elements can produce an artifact phenotype where none exists. This is demonstrated by distinct phenotypic effects that may be caused by different constructs used to target the same gene. Removal of the selection cassette is therefore recommended. Clean mutations can be achieved, either by the "hit-and-run" or "tag-and-exchange" methods, or by application of an exogenous recombinase.

"Hit-and-Run" and "Tag-and-Exchange" Methods

Several strategies have been demonstrated that can create specific substitutions. These strategies create subtle mutations in the gene, as opposed to disrupting it with a large insert. The "hit-and-run" method is based on selection of intrachromosomal recombination events that excise the vector sequence. In another strategy, termed "tag-and-exchange" (double replacement), a series of targeting procedures is performed. In the first stage, the gene is targeted with a vector that contains both positive and negative selections in the insert. In the second stage, a vector containing the mutation of choice is inserted by homologous recombination, selected by the loss of the negative selection marker.

Exogenous Recombinases

By introducing highly-efficient and specific exogenous recombination systems, both efficiency and tight control over the recombination event are achieved. The system most widely-used is the Cre recombinase from phage P1. Cre identifies a highly-specific 34 bp target sequence (*lox P*), and promotes reciprocal recombination between two such sites. The versatility of the system is such that, depending on the location of the *lox P* sequences, it can promote either reversible integration or excision, while retaining the recombination sites' integrity. Thus the Cre-*lox* system has been used for a variety of ends. These include the induction of chromosomal rearrangements, spatial or temporal inactivation of cellular genes by deletion, or activation of reporter genes. They also include multiple insertions into the same genomic site, and the creation of clean mutations devoid of exogenous regulatory sequences.

RNA Interference

While gene-targeting remains the most powerful method for the silencing of endogenous gene expression, post-transcriptional gene silencing has lately been characterized as a potent method of down-regulating specific genes. Double stranded RNA (dsRNA) molecules have been shown to initiate RNA interference (RNAi), causing sequence-specific mRNA degradation. Because RNAi does not entail any modification of the targeted gene itself, it is relatively simple to generate gene silencing using this method. Thus, RNAi inducing molecules can be directly introduced by transfection, or synthesized within the cells using expression vectors, either transiently or by stable integration.

The evolutionarily conserved machinery that underlies the phenomenon of homology-dependent gene silencing involves endonuclease (Dicer) processing of dsRNA into small interfering RNA (siRNA). siRNAs are 21- to 23-nt long fragments that, in turn, are incorporated into RISC, a multi-component nuclease, that uses the siRNA as a guide for the degradation of mRNA substrate. While the use of long dsRNA has been demonstrated to be effective, it is well-established that dsRNA within mammalian cells may induce a cellular response by interferon, thus generating non-specific effects. Direct use of siRNA has been shown to circumvent this problem.

In recent years, the RNAi technology has been extensively utilized for both mouse and human ES cells. The relative ease of designing siRNA molecules against specific gene targets, along with the straightforwardness of the knockdown experiment, is especially important in human ES cells for which double allele knockouts have not yet been achieved. The major hindering factor in the application of RNAi in human ES cells is the relatively low rate of transient transfection achieved, using standard methods, of up to 70%. Viral vectors can be used to achieve more efficient rates of genetic modification, and it has recently been proposed that nucleofection may also be at least as efficient. Nevertheless, siRNA is currently the most common method used by researchers wishing to down-regulate specific genes in human ES cells.

Conditional Genetic Modification

For many experimental applications, it could be worthwhile to perform conditional or controlled genetic modification. The means to achieve such modification are mainly through an inducible system, such as the tetracycline transactivator system, or by site-specific recombination, such as the previously described Cre-*lox*P system. Keeping the transgene from being expressed during the process of clone establishment can be essential in the case of deleterious or toxic genes. This is specifically important when transient transfection is problematic, either due to the relatively low transfection rates in human ES cells, or when long-term effects need to be examined. Additional applications could be the reversal of a phenotype conferred by a transgene—by its recombination-mediated removal or by the shutdown of the conditional system—or the conditional deletion of a genomic sequence.

CONCLUSIONS

One of the advantages of ES cells over other stem cell types currently under research is their accessibility for genetic manipulation. Progress so far indicates that genetic manipulation in human ES cells closely resembles that of their veteran mouse counterparts. Thus, most methods that have been used for the genetic manipulation of mouse ES cells have also been demonstrated in human ES cells. One handicap that human ES cells have is the problem of homozygotization of null alleles, which in the mouse is usually solved by breeding heterozygotes. Thus, it would seem that most of the genetic loss-of-function experiments in human ES cells will use RNAi. Possible uses for genetically-modified human ES cells could be the creation of cellular models for disease, studying various aspects of early human development, transplantation medicine, and many more. Thus, in the near future, a large number of studies using genetic manipulation techniques in human ES cells are foreseeable.

KEY WORDS

DNA transfection The introduction of foreign DNA molecules into cultured cells.

Gene-targeting The modification of a gene in a specific and directed manner through homologous recombination with a DNA segment. Possible applications include the elimination of gene expression, or correction of a mutation in a gene.

Homologous recombination The substitution of one DNA segment by another, based on exact or near identity between them.

Negative selection The application of growth conditions eliminating those cells expressing the selection marker.

Positive selection The application of specific growth conditions causing selective elimination of cells that do not contain an exogenous genetic element (selection marker) conferring resistance.

RNA interference (RNAi) The process of post-transcriptional down-regulation of gene function through the use of non-coding RNA molecules, such as small interfering RNA (siRNA).

Stable transfection Transfection in which foreign DNA is incorporated into the host genome, generating a long-term genetic modification.

Transient transfection Transfection in which plasmid DNA is not physically incorporated into the genome, and is retained as an episome, generating short-term gene expression of the foreign DNA.

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Chapter 46

Homologous Recombination in Human Embryonic Stem Cells

Thomas P. Zwaka and James A. Thomson

> Biologists have long attempted by chemical means to induce in higher organisms predictable and specific changes as hereditary characters.
>
> Avery, 1944

INTRODUCTION

Homologous recombination provides a precise mechanism for defined modifications of genomes in living cells, and has been used extensively with mouse embryonic stem cells to investigate gene function and to create mouse models of human diseases. The ability to modify the mouse genome in the intact mouse in a predetermined manner has revolutionized biomedical research involving mice. Two breakthroughs have made this possible: the derivation of embryonic stem (ES) cells; and the identification of conditions for recombination between incoming DNA and the homologous sequences in the mammalian chromosome.

Recently, human ES cell lines have been derived from pre-implantation embryos. Human ES cells can be maintained for a prolonged period of culture, while retaining their ability to form extraembryonic tissues and advanced derivatives of all three embryonic germ layers. Human ES cells provide a new model for understanding human development, a renewal source of human cells either for *in vitro* studies or for transplantation therapies, and new *in vitro* models for understanding human disease.

Differences between mouse and human ES cells delayed the development of homologous recombination techniques in human ES cells. First, high, stable transfection efficiencies in human ES cells have been difficult to achieve and, in particular, electroporation protocols established for mouse ES cells do not work in human ES cells. Second, in contrast to mouse ES cells, human ES cells clone inefficiently from single cells, making screening procedures to identify rare homologous recombination events difficult. We have recently developed protocols to overcome these problems. Here we describe how these protocols have allowed us to successfully target a ubiquitously expressed gene (*HPRT1*), an ES cell-specific gene (*POU5F1*), and a tissue-specific gene (*tyrosine hydroxylase*, *TH*).

TARGETED ABLATION OF THE HPRT1 GENE AS A TOOL TO OPTIMIZE HOMOLOGOUS RECOMBINATION EFFICIENCY

The *HPRT1* gene is located on the X-chromosome, so a single homologous recombination event can lead to complete loss of function in XY cells. *HPRT1*-deficient cells can be selected based on their resistance to 2-amino-6-captopurine (6-TG), and thus, the frequency of homologous recombination events is easy to estimate. It was because of these properties that the *HPRT1* gene played such an important role in the initial development of homologous recombination in mouse ES cells.

Therefore, we designed a gene-targeting vector that was able to ablate parts of the human *HPRT1* gene, and we used this system to optimize transfection protocols in human ES cells. The gene-targeting vector was constructed by substituting the last three exons (exons 7, 8, and 9) of the *HPRT1* gene by a *neo* resistance cassette under the thymidine-kinase (tk) promoter control (Figure 46-1A). This cassette was flanked in the 5″ region by a 10-kb homologous arm, and by a 1.9-kb homologous arm in the 3″ region. At the end of the 3″ homologous arm, the *tk* gene was added for negative selection.

Previously-described problems with nonisogenic genomic DNA in mouse experiments encouraged us to use homologous DNA that was isogenic to the particular human ES cell line we wanted to target (H1 subclone 1). This DNA was obtained by long-distance, high-fidelity, genomic polymerase chain reaction (PCR), and subcloned into a PCR-cloning vector.

For human ES cells, the best chemical reagents yield stable (drug-selectable) transfection rates of about 10^{-5},

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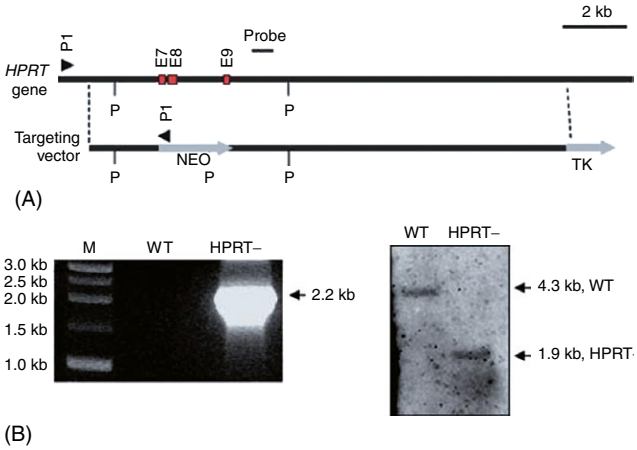


FIGURE 46-1 Knockout of the *HPRT1* gene. (A) Genomic organization of the human *HPRT1* gene and the gene-targeting vector. 3″ probe for Southern blot analysis is shown, E = exon; P = PstI; P1 = primer pair; TK = thymidine-kinase. (B) PCR analysis of ES cell lines (HPRT = knockout; WT = wild-type cells, M = marker) with P1 (left) and Southern blot analysis with dedicated probe and PstI digest (right).

TABLE 46-1 Numbers of Colonies Obtained by Positive and Negative Selection and Targeted Events in the *HPRT* Gene Locus (from 1.5×10^7 Electroporated Human ES Cells)

Selection Procedure	ExGen 500	Fugene 500	Electroporation
G418	130	261	350
G418 and gancyclovir	35	61	50
G418 and 6-TG	0	0	7

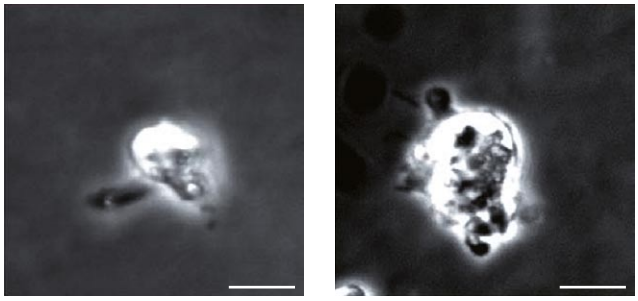


FIGURE 46-2 Size comparison of mouse (left) and human (right) embryonic stem cells (bar = 10 mm).

and mouse ES cell electroporation procedures yield rates that are significantly lower. Therefore, we started to test our *HPRT1* gene-targeting vector system using FuGene-6 and ExGen 500, two chemical transfection reagents commonly used in human ES cell work.

Two days before transfection, human ES cells (H1 subclone 1) were trypsinized and plated on Matrigel at a density of 200 000 cells/6-well plate, and cultured with fibroblast-conditioned medium in six-well plates. Transfection, using FuGene-6 or ExGen 500, was performed according to the manufacturers' recommendations. Briefly, the vector was co-incubated with the plasmid (2 mg DNA and 6 ml FuGene-6, or 3 mg DNA and 10 ml ExGen 500), and added to the supernatant of each well of the six-well plate. In experiments using ExGen 500, we gently centrifuged the six-well plate after adding the DNA/ExGen mixture (300 g for five minutes). The medium was changed four hours after transfection, G418 selection was started two days after transfection, and two days later, selection with 6-TG (1 mM) and/or GANC (1 mM) was begun.

Although G418 (130 versus 261) and gancyclovir-resistant (35 versus 61) clones were obtained for both transfection reagents, none of these was G418- and 6-TG resistant ($HPRT^-$), indicating that none was the result of homologous recombination (see Table 46-1). These results are consistent with the observation that transfection using lipid (FuGene-6) and cationic (ExGen 500) reagents results in inefficient homologous recombination in other mammalian cell types, and that physical methods of introducing DNA, in general, are more effective. A possible explanation is that physical transfection induces a cellular stress reaction, and activates the recombination machinery to a greater extent than do chemical transfection reagents.

Many parameters influence the transfection rate for electroporation and, usually, the procedure has to be optimized for each cell line. This is critical for human ES cells, as they are very sensitive and difficult to grow from individual cells. Indeed, the difference between the diameter of human and mouse ES cells suggests that successful protocols will be significantly different (Figure 46-2). This was confirmed by an experiment, using a typical mouse ES cell protocol, which clearly showed the overall transfection rate to be too low in human ES cells to expect recovery of homologous-recombinant clones (Table 46-1). In our hands, electroporation using a typical mouse ES cell protocol (220 V, 960 mF electroporation in PBS) yielded a stable transfection rate in human ES cells that was lower than $\sim 10^{-7}$.

We adapted electroporation parameters for human ES cells. To increase plating efficiency, we removed ES cell colonies from the culture dish as intact, small clumps, and plated them out at a high density following electroporation. To increase survival, we used culture medium instead of PBS as an electroporation buffer, and performed electroporation at room temperature. Finally, we changed the physical parameters of the electrical field during electroporation. Applying this modified protocol, we were able to achieve stable, G418-resistant transfection rates that were 100-fold (or more) higher than standard mouse ES cell electroporation procedures.

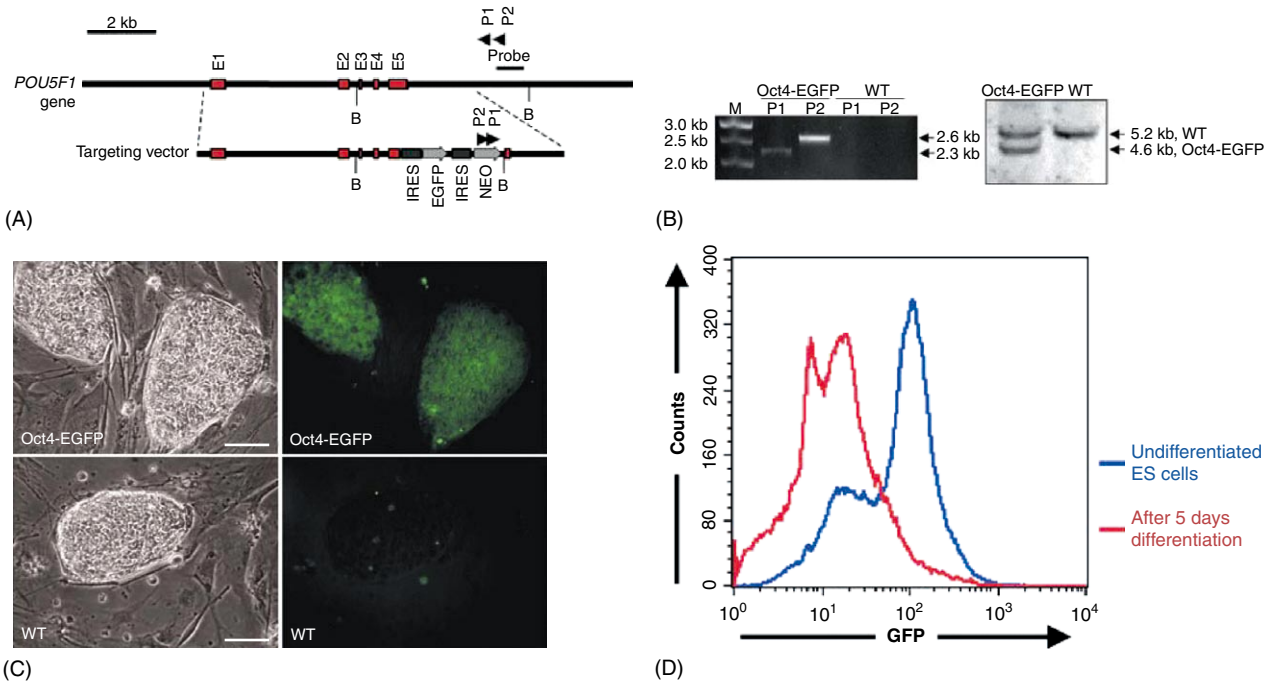


FIGURE 46-3 Knockin of an IRES-EGFP-IRES-*neo* cassette into the 3′ UTR of the Oct4-coding gene *POU5F1*. (A) Genomic structure of the human *POU5F1* gene and the gene-targeting vector. 3′ probe for Southern blot analysis is shown, E = exon; B = BamHI; P1 and P2 = primer pairs 1 and 2. (B) PCR analysis of ES cell lines (Oct4-EGFP = heterozygous knockin; WT = wild-type cells, M = marker) with P1 and P2 (left) and Southern blot analysis with dedicated probe and BamHI digest (right). (C) Fluorescence microscopy (right) and phase contrast (left) of Oct4 knockin and wild-type colonies, bar = 25 mm. (D) Flow cytometry of Oct4 knockin, undifferentiated (EGFP-positive) ES cells (blue), and their differentiated derivates after five days of differentiation (red).

In order to remove ES cells as clumps, we treated cultures with collagenase IV (1 mg/ml, Invitrogen) for seven minutes, washed with media, and resuspended the cells in 0.5 ml of culture media (1.5–3.0 × 10^7 cells). Just prior to electroporation, 0.3 ml of phosphate-buffered saline (PBS, Invitrogen) containing 40 mg linearized targeting vector DNA was added. Cells were then exposed to a single, 320 V, 200 mF pulse at room temperature, using the BioRad Gene Pulser II (0.4 cm gap cuvette). Cells were incubated for 10 minutes at room temperature, and were plated at high density (3.0 × 10^7 cells per 10 cm culture dish) on Matrigel. G418 selection (50 mg/ml, Invitrogen) was started 48 hours after electroporation. After one week, G418 concentration was doubled, and 6-TG selection (1 mM, Sigma) was started. After three weeks, surviving colonies were picked, transferred to 48-well plates, and analyzed individually by PCR, using primers specific for the *neo* cassette and for the *HPRT1* gene just upstream of the 5′ region of homology, respectively. PCR-positive clones were rescreened by Southern blot analysis using PstI-digested DNA and a probe 3′ of the *neo* cassette. After transfection of 1.5 × 10^7 human ES cells, 350 G418-resistant clones were obtained. Among them, 50 were gancyclovir-resistant, and of those, seven were also 6-TG-resistant, suggesting successful homologous recombination (Table 46-1). PCR and Southern blotting (Figure 46-2B) confirmed that homologous recombination had occurred in all of the 6-TG-resistant clones. Electroporation of human ES cells by a DNA construct containing a *neo* resistance cassette, under the control of the *tk* promoter, yielded a stable transfection rate of 5.6 × 10^{-5}.

OCT4 EGFP/*NEO* KNOCKIN

A useful application for "knockins" in human ES cells will be to generate cell lines with a selectable marker introduced into a locus with a tissue-specific expression pattern. Such knockins can be used, for example, to purify a specific ES cell-derived cell type from a mixed population. To test this approach, we introduced two reporter genes into the Oct4 coding gene *POU5F1* by homologous recombination. Oct4 belongs to the POU (Pit, Oct, Unc) transcription factor family, is expressed exclusively in the pluripotent cells of the embryo, and is a central regulator of pluripotency. We introduced two promoterless reporter/selection cassettes into the 3′ untranslated region (UTR) of the human Oct4 gene (Figure 46-3A). The first cassette contained an internal ribosomal entry site (IRES) sequence of the encephalomyocarditis virus, and a gene encoding the enhanced green fluorescence protein (EGFP). The second cassette included the same IRES sequence, and a gene-encoding neomycin resistance (*neo*). The cassettes were flanked by two homologous arms (6.3-kb in the 5′ direction, and 1.6-kb in the

3′ direction). After electroporation of 1.5×10^7 human ES cells with the linearized targeting vector, we obtained 103 G418-resistant clones. PCR and DNA Southern blotting demonstrated that 28 of these clones (27%) were positive for homologous recombination (Figure 46-3B). Using a second targeting vector with a longer 3′ homologous arm, the rate of homologous recombination increased to almost 40% (22 homologous clones out of 56 G418-resistant clones). Similar transfection experiments, using FuGene-6 with the same Oct4 gene-targeting vector, produced 11 G418-resistant clones, none of which resulted from homologous recombination.

Human ES cells with the Oct4 knockin expressed EGFP. After induction of differentiation with various stimuli, EGFP expression was turned off, indicating a down-regulation of Oct4. Both drug selection and flow cytometry for EGFP expression allowed purification of undifferentiated ES cells from a mixed, partially-differentiated cell population. These properties will make the knockin cell lines useful for studying Oct4 gene expression during differentiation, *in vitro*, and optimizing culture conditions for human ES cells.

TH EGFP KNOCKIN

Because Oct4 and HPRT are both expressed in human ES cells, we next asked if it is possible to target a gene that is not expressed in ES cells. TH is the rate-limiting enzyme in the synthesis of dopamine, and it is one of the most common markers used for dopaminergic neurons. In order to achieve bicistronic expression of both TH and EGFP, we constructed a gene-targeting vector that introduces an IRES-EGFP reporter gene cassette into the 3′ UTR region in the last exon of the *TH* gene (Figure 46-4A). The cassette is flanked by a short homologous arm 5′ of this exon (1.4-kb) and a long homologous arm in the 3′ region of the last exon (8-kb). The long arm follows the gene for *tk* for negative selection of random integrated, stable transfected clones. Between the long homologous arm and the IRES-EGFP cassette, we cloned a PGK-driven *neo* resistance cassette flanked by two *loxP* sites. After transfection of 6.0×10^7 human ES cells (cell line H9), we obtained five PCR-confirmed, homologous-recombinant clones after double selection for the positive selection marker *neo* with G418, and the negative selection marker TK with gancyclovir (Figure 46-4B).

The positive selection marker in this experiment was a *neo* cassette under the PGK promoter. As this cassette is still present in the knockin cell line, it could interfere with the normal expression of the *TH* gene itself and the IRES-EGFP reporter gene. To delete the *neo* cassette, we transiently transfected two of the TH-EGFP knockin cell lines with a plasmid containing the phage recombinase Cre under the control of the EF1a promoter. We transfected with FuGene-6, and used the protocols described above. The cDNA of Cre was followed by an IRES-EGFP cassette. After transient transfection with this plasmid, Cre overexpressing cells could be easily identified by EGFP expression. Those EGFP-positive cells were purified by fluorescence-activated cell sorting (FACS). Individual

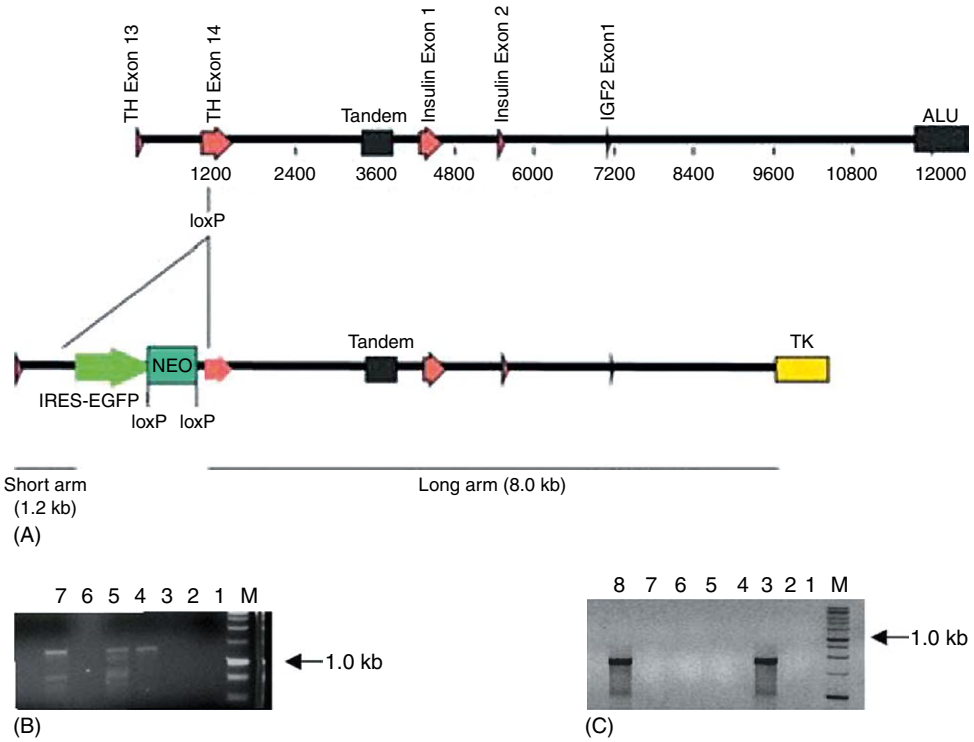


FIGURE 46-4 Knockin of an IRES-EGFP cassette into the 3′ UTR of the *TH* gene. (A) Partial structure of the human *TH* gene and the gene-targeting vector. (B) Some of the G418/GANC resistant clones (4, 5, and 7) are positive in the screening PCR, indicating successful homologous recombination. (C) After Cre transfection, two of the eight screened clones (3 and 8) show the PCR product that indicates a positive recombination event.

clones were analyzed for successful recombination of the two *loxP* sites, and two clones were identified that have the *neo* cassette excised by PCR (Figure 46-4B). The clones were confirmed to have a normal karyotype.

We performed the *TH* gene targeting on cell line H9, using a targeting construct that was homologous to genomic DNA from the cell line H1 (see Table 46-2), suggesting that isogenic DNA is not absolutely required to obtain homologous recombination in human ES cells.

CONCLUSIONS

The overall targeting frequencies for the three genes discussed here suggest that homologous recombination is a broadly applicable technique in human ES cells (Table 46-2). The homologous recombination frequencies are roughly comparable to those observed for mouse ES cells and suggest that, although successful transfection strategies differ between human and mouse ES cells, homologous recombination itself may be similar. We also demonstrated that the Cre/lox system can be used to excise specific sequences from human ES cells.

Homologous recombination in human ES cells will be important for studying gene function *in vitro*, and for lineage selection. It is a powerful approach for understanding the function of any human gene. For therapeutic applications in transplantation medicine, controlled modification of specific genes should be useful for purifying specific ES cell-derived, differentiated cell types from a mixed population, and for altering the antigenicity of ES cell derivatives. In addition, it should be possible to give cells new properties (such as viral resistance) to combat specific diseases. Homologous recombination in human ES cells might also be used for recently described approaches combining therapeutic cloning with gene therapy.

In vitro studies will be particularly useful for learning more about the pathogenesis of diseases where mouse models have proven inadequate. For example, HPRT-deficient mice fail to demonstrate a Lesch–Nyhan-like phenotype. *In vitro* neural differentiation of HPRT− human ES cells, or transplantation of ES cell-derived neural tissue to an animal model, could help in understanding the pathogenesis of Lesch–Nyhan syndrome. Another good example of where human ES cells and homologous recombination will be useful is in understanding the function of the human heart. Specific mutations and polymorphisms that predispose people to an increased risk of sudden death syndrome or severe arrhythmia at a young age have been identified. Mice generated from ES cells in which ion channel genes have been modified by homologous recombination often have a normal heart, again reflecting basic, clinically-significant species differences. With homologous recombination, one could generate human ES cell lines bearing mutations or polymorphisms in specific ion channels, and use ES cell-derived cardiomyocytes to better understand the effects of the mutations on the physiology of the heart. A panel of ion channel polymorphisms should be extremely useful for screening drugs for toxic side-effects on the heart prior to clinical trials. Thus, homologous recombination in human ES cells would be useful for numerous *in vitro* models of human disease.

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KEY WORDS

Embryonic stem cells Pluripotent cell lines derived from mammalian blastocysts.

TABLE 46-2 Comparison of Gene-targeting Frequencies in Different Experimental Setting in Human Embryonic Stem Cells (Data Normalized to 1.5×10^8 Electroporated Human ES Cells). Homologous DNA was Isogenic to H1 Genomic DNA (ND = Not Determined)

Selection Procedure	Old Mouse ES Cell Protocol	*HPRT1* in H1.1	Oct4 Short Vector in H1.1	Oct4 Long Vector in H1.1	Oct4 Short Vector in H9	*TH* in H9 ND
G418	1.0	230	69	37	48	3.8
G418 and gancyclovir	ND	3.3	ND	ND	ND	1.3
Homologous recombinants	ND	5	19	15	2	

Homologous recombination The reciprocal exchange of sequences between two homologous DNA strands. It is widely used to introduce targeted and specific modifications in prokaryotic and eukaryotic genomes.

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Chapter 47

Surface Antigen Markers

Jonathan S. Draper and Peter W. Andrews

INTRODUCTION

The accessibility of molecules on the surface of cells makes them exceptionally convenient markers for characterizing cell types. Often recognized as antigens by specific antibodies, some of these surface molecules are common to many cells, but others show highly restricted patterns of expression characteristic of particular cell types. Boyse and Old (1996) called such antigens with restricted expression "differentiation antigens," and the patterns of expression of these markers, or "surface antigen phenotypes," have been widely used for analyzing cell relationships in the hematopoietic and lymphoid systems. Initially, polyclonal sera, produced by cross-immunizing mice of different strains bearing alternate alleles of specific surface antigens, were used to define subsets of lymphocytes with different functional characteristics. With the subsequent development of monoclonal antibodies, a wide array of so-called cluster determinant markers were characterized for defining cells of different lineages within the hematopoietic system of both mice and humans, and for identifying the pluripotent hematopoietic stem cells from bone marrow.

Although many surface antigens are informative in certain defined contexts, many are frequently reused at different temporal and special points during the life of an organism. An apposite example is the expression of stage-specific embryonic antigen-3 and -4 (SSEA3 and SSEA4) on cells of cleavage and blastocyst stage human embryos, and separately as part of the P-blood group system on adult red blood cells. Indeed, it can be difficult to establish whether expression of an antigen is restricted to a specific cell type. Thus, caution is required when interpreting the presence or absence of a particular antigen and extrapolating to the identification of the cell type, or to the definition of lineage relationships between cell types. That two cell types express a common antigen is not evidence that they are related by a common lineage, although this erroneous argument is sometimes encountered. Nevertheless, surface antigens can provide powerful tools for analyzing and sorting cells that have particular characteristics within specific contexts. Added flexibility is provided by the ability to use antibodies directed to surface antigens for labeling and sorting live cells into discrete populations that retain viability.

CELL-SURFACE EMBRYONIC ANTIGENS OF THE LABORATORY MOUSE

Building on the work of Boyse and Old with differentiation antigens of the adult hematopoietic system, Artzt and her colleagues in the Institut Pasteur proposed that immunization of inbred adult mice with syngeneic embryonic cells should yield an antiserum that would recognize those antigens that are expressed only in the embryo and not in the adult. They immunized 129 mice with F9 embryonal carcinoma (EC) cells derived from a teratocarcinoma of a 129 male mouse. The resulting antiserum did react specifically with EC cells, and some early embryonic cells, notably those of cleavage-stage embryos and the inner cell mass (ICM) of the blastocyst, but not with postblastocyst-stage embryos, differentiated derivatives of EC cells, or various cells of adult origins. This pattern of reactivity supported the notion that embryo-specific surface antigens exist, and contributed to the argument that EC cells from teratocarcinomas are malignant counterparts of ICM cells, the "stem cells" of the early embryo.

Although some progress was made in characterizing the F9 antigen as a high-molecular-weight glycoprotein, the complexity and variability of such sera impeded detailed study. The development of monoclonal antibodies provided a route to much better-defined reagents. Of these, the best known is the monoclonal antibody MC480, produced from a BALB/c mouse immunized with EC cells from a 129 teratocarcinoma. This antibody defines SSEA1, which shows a similar pattern of expression to the F9 antigen and, most likely, the epitope recognized by MC480 is a significant component of the F9 antigen.

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SSEA1 was identified as a glycosphingolipid, and the epitope was shown to be the Lewis-X (Le^x) haptene. However, the epitope can also be carried by high-molecular-weight glycoproteins. Like the F9 antigen, SSEA1 was found to be expressed by blastomeres of late-cleavage and morula-stage embryos, by cells of the ICM, and by trophectoderm. It is also characteristically expressed by mouse EC and embryonic stem (ES) cells, and has become one of the hallmarks for identifying these undifferentiated stems cells; their differentiation is typically characterized by disappearance of this antigen. Nevertheless, variant EC lines lacking expression of SSEA1 have been isolated, so SSEA1 expression is not, in itself, a requirement for a pluripotent EC–ES phenotype.

Another embryonic antigen, SSEA3, was defined by a monoclonal antibody, MC631, raised by immunizing rats with four-cell-stage mouse embryos. This antigen was found to be expressed by cleavage embryos of most strains of mice, but then it disappears from the ICM of the blastocyst to reappear on the primitive endoderm. Its reactivity was thus the reciprocal of SSEA1 expression. It also proved to be a glycosphingolipid, but with a globoseries core structure rather than with the lactoseries core of SSEA1 (Table 47-1). A further antigen in the series, SSEA4, defined by monoclonal antibody MC813-70, shows an expression pattern similar to that of SSEA3, and is a globoseries glycosphingolipid similar to the SSEA3-reactive lipid, but with a terminal sialic acid.

Although SSEA3 and SSEA4 are not expressed by murine EC cells and embryonic cells of the ICM, the Forssman antigen is expressed by these cells. The Forssman antigen is also a globoseries glycolipid, but it has a terminal galactosamine residue that can be added to globoside in rodent, but not in human, cells.

Capitalizing on these studies of teratocarcinoma-derived EC cells, in 1981 Evans and Kaufman and, independently, Martin demonstrated that it is possible to derive cell lines by explanting the ICM of blastocysts onto feeder cell layers *in vitro*, under conditions used in some of the early derivations of EC cell lines. Subsequently, embryonic germ cell lines closely resembling EC and ES cells were established. These embryo-derived cells closely resembled EC cells morphologically and in developmental potential. They also expressed similar markers, including SSEA1.

TABLE 47-1 Glycolipid Antigens of Mouse and Human Embryonal Carcinoma and Embryonic Stem Cells

Antigen	Oligosaccharide structure
	Globoseries glycolipids
SSEA3	Galb1 Æ 3GalNAcb1 Æ 3Gala1 Æ 4**Galb1** Æ **4Glcb1** Æ **Cer**
SSEA3, SSEA4	NeuNAca Æ 3Galb1 Æ 3GalNAcb1 Æ 3Gala1 Æ 4**Galb1** Æ **4Glcb1** Æ **Cer**
Forssman	GalNAca1 Æ 3GalNAcb1 Æ 3Gala1 Æ 4**Galb1** Æ **4Glcb1** Æ **Cer**
	Lactoseries glycolipids
SSEA1	Galb1 Æ 4GlcNAcb1 Æ 4**Galb1** Æ **4Glcb1** Æ **Cer** 3 ≠ Fucal
	Ganglioseries glycolipids
GT3 (A2B5)	(9-0-acetyl) NeuNAca2 Æ 8NeuNAca2 Æ 3**Galb1** Æ **4Glcb1** Æ **Cer**
9-O-acetyl GD3 (ME311)	NeuNAca2 Æ 8NeuNAca2 Æ 8NeuNAca2 Æ 3**Galb1** Æ **4Glcb1** Æ **Cer**

All of these glycosphingolipid antigens are synthesized by extension from a common precursor, lactosylceramide. The likely parts of these structures comprising the epitopes recognized by the different antibodies are highlighted. The enzymes responsible for the addition of the third sugar residues (galactosyl, glucosaminyl-, and sialyltransferase) appear to regulate the rate-limiting step that controls which synthetic pathway predominates. Human EC and ES cells are characterized by expression of the glycolipid antigens SSEA3 and SSEA4, and not the lactoseries antigen SSEA1 (Le^x). By contrast, mouse EC and ES cells are typically characterized by their expression of SSEA1. Mouse cells also express the globoseries Forssman antigen, which is not synthesized by human cells. Thus, despite the apparent distinction between mouse and human EC and ES cells in terms of their terminal sugars that comprise the different epitopes, the undifferentiated cells of both species do seem to share in common the expression of globoseries core structures.

HUMAN EMBRYONAL CARCINOMA AND EMBRYONIC STEM CELLS

Initially, it was assumed that human EC cells would closely resemble mouse EC cells; in the first studies of cell lines derived from human testicular cancers, it was reported that human EC cells, like murine EC cells, were F9 antigen-positive. However, on more detailed examination, it was established that, although human EC cells closely resemble mouse EC cells in their general morphology and growth patterns, they do not express some of the markers characteristic of mouse EC cells; in particular, they lacked SSEA1. By contrast, human EC cells *in vitro*, and in tumors, express SSEA3 and SSEA4.

The functional significance of these glycolipid antigens, which provide excellent markers of the undifferentiated EC and ES cells in both mice and humans, remains obscure. They appear to be strongly developmentally-regulated: SSEA1 expression is rapidly down-regulated on differentiation of mouse EC cells. In human EC and ES cells, not only is differentiation marked by down-regulation of the globoseries antigens SSEA3, but at least on some derivative cells, these antigens are replaced by SSEA1 or by ganglioseries antigens, notably ganglioside GT3 recognized by antibody A2B5 (Table 47-1). This change

in expression seems to relate to switching expression of the glycosyl transferases responsible for synthesis of the globo-, lacto-, and ganglioseries core oligosaccharide structures. It might be that the difference between mouse and human EC–ES cells is smaller than at first sight, since globoseries glycolipids are prominent in both species. The differences are in terminal modifications to yield Forssman in the mouse, and SSEA3 and SSEA4 in humans (Table 47-1). SSEA1 mouse EC cells and SSEA3/4 human EC cells have been reported, although at least in the case of the human cells, this is a consequence of changes to terminal modification rather than of expression of core glycolipid structures. However, a small number of humans, those with the pk and pp blood groups, are unable to synthesize the elongated globoseries structures, and do not express SSEA3 or SSEA4. Thus, lack of these structures is evidently not detrimental to embryonic development. More tellingly, it is possible to eliminate all glycolipid synthesis with the inhibitor of glucosylceramide synthetase, PDMP: NTERA2 human EC cells still survive and differentiate in the presence of this inhibitor, and embryos of medaka fish develop normally despite near-elimination of glycolipid synthesis. Interestingly, however, pk and pp women frequently have high rates of early spontaneous abortions, which might be caused by an immune response to embryonic antigens, perhaps SSEA3, SSEA4, or both.

A different series of antigens of human EC and ES cells comprises the keratan sulfates, different epitopes of which are recognized by several antibodies, most notably TRA-1-60, TRA-1-81, GCTM2, K4, and K21. Of these antigens, TRA-1-60 is dependent upon a terminal sialic acid, and GCTM2 has been reported to recognize the internal protein structure. Unlike SSEA3 and SSEA4, expression of these antigens is unaffected by PDMP.

Human ES cells closely resemble human EC cells in expression of these antigens; they are generally SSEA3+, SSEA4+, TRA-1-60+, TRA-1-81+, GCTM2+, and SSEA1−. Similarly, the ICM cells of human blastocyst stage embryos are SSEA3+, SSEA4+, TRA-1-60+, and SSSEA1−, so human EC and ES cells resemble human ICM cells, as mouse EC and ES cells resemble mouse ICM cells, even though these cells differ between the two species.

Other surface antigens that appear to be characteristic of human EC and ES cells include Thy1 and the tissue-nonspecific form of alkaline phosphatase, which can be readily recognized by the monoclonal antibodies TRA-2-49 and TRA-2-54.

The differentiation of human EC and ES cells is characterized by the loss of expression of all of these various markers of undifferentiated cells. At the same time, various antigens appear. SSEA1, which seems to be absent from undifferentiated human EC and ES cells, frequently appears on differentiation. At least some of the cells that express this antigen may belong to the trophoblastic lineage. Ganglioside antigens, notably GT3, recognized by antibody A2B5, and GD2 and GD3, recognized by antibodies VIN2PB22 and VINIS56, all appear on subsets of cells on human EC and ES differentiation, and most likely identify cells of the neural lineages.

One set of antigens that can cause confusion in comparisons of mouse and human EC and ES cells is the class 1 major histocompatibility antigens, H-2 in mice and HLA in humans. H-2 antigens are not expressed by murine EC cells, although they do appear on differentiation. HLA is generally expressed by human EC and ES cells, and may show a transient down-regulation on differentiation. HLA class 1 antigen expression is also strongly induced in human EC and ES lines by interferon-g, although an antiviral response does not occur.

SUMMARY

Several surface marker antigens that show developmental regulation are found on both mouse and human EC and ES cells, although there are significant species differences in the particular antigens expressed (Table 47-2), and a greater array of such markers is available for human cells. These antigens can provide important tools, both for identifying undifferentiated stem cells and for monitoring their

TABLE 47-2 Differential Expression of Characteristic Surface Marker Antigens of Mouse and Human Embryonal Carcinoma and Embryonic Stem Cells

Antigen	Mouse	Human	Comments
Forssman	Yes	No	Globoseries glycolipid
SSEA1 (Le^x)	Yes	No	Lactoseries glycolipid
SSEA3	No	Yes	Globoseries glycolipid
SSEA4	No	Yes	Globoseries glycolipid
TRA-1-60	No	Yes	Keratan sulfate
TRA-1-81	No	Yes	Keratan sulfate
GCTM2	No	Yes	Keratan sulfate
Liver alkaline phosphatase (L-ALP)	Yes	Yes	No antibody is available for murine ALP, but human L-ALP is recognized by antibodies TRA-2-54 and TRA-2-49
MHC Class 1	No	Yes	H-2 in the mouse; HLA in humans

differentiation. A workshop comparison of the antigens expressed by a large panel of human EC cells suggested that SSEA3, an antigen rapidly lost as human EC cells differentiate, may be one of the more sensitive indicators of the undifferentiated state. However, a similar comparison of antigen expression by a panel of human ES lines remains to be undertaken. Furthermore, caution is warranted; some human EC cell sublines may lack SSEA3 expression, but retain the expression of other markers of the undifferentiated state and display pluripotency, as evidenced by an ability to differentiate *in vivo* and *in vitro*. Also, many of the key markers so far defined have oligosaccharide epitopes, the expression of which depends on a variety of complex interacting factors, not merely the expression or the lack of expression of specific glycosyl transferases. There remains, therefore, considerable scope for the identification of additional surface antigens for identifying and monitoring undifferentiated human ES cells.

APPENDIX: METHODS

Indirect Immunofluorescence and Flow Cytometry

1. Harvest cells to obtain a single-cell suspension. Use any standard protocol; typically, 0.25% trypsin in 1 mM EDTA, in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), is used. After pelleting, resuspend the cells in either HEPES-buffered medium or wash buffer (5% fetal calf serum in PBS plus 0.1% sodium azide) to 10^7 cells per milliliter. (If there are fewer cells, then 2×10^6 per ml can conveniently be used.)

2. Choose antibodies and dilute as appropriate in wash buffer.

3. Distribute the primary antibodies (Table 47-3), diluted appropriately after a preliminary titration, at 50 ml per well of a round-bottom 96-well plate, one well for each assay point. To prevent carryover from one well to another, it is good practice to use every other well of the plate. As a negative control, we use the antibody from the original parent myeloma of most hybridomas, namely, P3X63Ag8. However, others may prefer a class-matched nonreactive antibody if one is available or there is no first antibody.

4. Add 50 ml of cell suspension (i.e., $10^5 - 5 \times 10^5$ cells) to each 50 ml of antibody.

5. Seal the plate by covering it with a sticky plastic cover, ensuring that each well is sealed, and incubate at 4ºC, with gentle shaking, for 30 to 60 minutes.

6. Spin the plate at 280 × g for three minutes, using microtiter plate carriers in a tissue culture centrifuge. Check that the cells are pelleted, and remove the plastic seal using a sharp motion, but holding the plate firmly to avoid disturbing the cell pellet. Dump the supernatant

TABLE 47–3 Common Antibodies Used to Detect Antigens Expressed by Human Embryonal Carcinoma and Embryonic Stem Cells

Antibody	Antigen	Antibody Species and Isotype	Remarks
MC631	SSEA3	Rat IgM	Undifferentiated hES–EC
MC480	SSEA1 (Le^x)	Mouse IgM	Differentiated hES–EC; including trophoblast
A2B5	GT3	Mouse IgM	Neural
ME311	9-0-acetylGD3	Mouse IgMG	Neural
VINI556	GD3	Mouse IgM	Neural
VIN2PB22	GD2	Mouse IgM	Neural
MC813-70	SSEA4	Mouse IgG	Undifferentiated hES–EC
TRA-1-60	TRA-1-60	Mouse IgM	Undifferentiated hES–EC
TRA-1-81	TRA-1-81	Mouse IgM	Undifferentiated hES–EC
TRA-2-54	L-ALP	Mouse IgG	Undifferentiated hES–EC
TRA-2-49	L-ALP	Mouse IgG	Undifferentiated hES–EC
TRA-1-85	Ok[a]	Mouse IgG	Panhuman
BBM1	b2-microglobulin	Mouse IgG	HLA
W6/32	HLA-A, -B, -C	Mouse IgG	HLA

by inverting the plate with a rapid downward movement; blot the surface and turn the plate over. Provided that this is done in a single movement without hesitation, the cells remain as pellets at the bottom of the wells. If there are any concerns about pathogens, or any other contaminant of a culture, supernatants can be removed by aspiration rather than dumping.

7. Wash the cells by adding 100 ml of wash buffer to each well, seal, and agitate to resuspend the cells. Spin down as described previously. After removing the supernatant, repeat with two more washes.

8. After the third wash, remove the supernatant, and add 50 ml of fluorescent-tagged antibody, previously titered and diluted in wash buffer, to each well. We routinely use FITC-tagged goat–antimouse IgM or antimouse IgG, as appropriate, to the first antibody. Antimouse IgM, but not antimouse IgG, usually works satisfactorily with MC631 (a rat IgM). Affinity-purified and/or $F(ab')_2$ second antibodies may be used if required to eliminate background.

9. Seal the plate as described previously, and repeat the incubation and washings as before.

10. Resuspend the cells, about 5×10^5 per milliliter, in wash buffer and analyze in the flow cytometer. The precise final cell concentration will depend on local operating conditions and protocols.

NOTES

All antibodies, both primary and secondary, should be pretitered on a standard cell line to determine optimal concentrations. For monoclonal antibodies, we typically find that the dilution for ascites is between 1:100 and 1:1000, and for hybridoma supernatants, it is between 2X and 1:10.

A useful antibody is TRA-1-85 (Table 47-3). This antibody recognizes an antigen, Ok[a], which appears to be expressed by all human cells, but not by mouse cells. It is therefore valuable as a tool for distinguishing human ES cells from the mouse feeder cells on which they are commonly grown.

Fluorescence-activated Cell Sorting (FACS)

1. Harvest the hES cells using trypsin-EDTA, as before, for analysis. Note, however, that lower concentrations of trypsin (0.05%) greatly improve cell viability. The cells should be pelleted in aliquots—10^7 cells per aliquot is convenient.

2. Resuspend the cells in primary antibody, diluted in medium without added azide, as determined by prior titration (100 ml per 10^7 cells). The primary and secondary antibodies should be sterilized using a 0.2 micron cellulose acetate filter.

3. Incubate the cells, with occasional shaking, at 4ºC for 20–30 minutes.

4. Wash the cells by adding 10 ml medium, and pellet by centrifugation at 200 g for five minutes; repeat this wash step once.

5. Remove supernatant and gently flick to disperse the pellet. Add 100 ml of diluted secondary antibody per 10^7 cells and incubate, with occasional shaking, at 4ºC for 20 minutes.

6. Wash the cells twice as described previously. After the final wash, resuspend the cells in medium at 10^7 cells per milliliter. Sort the cells using the flow cytometer according to local protocols. If the cells are to be cultured after sorting, sort into hES medium supplemented with antibiotics.

NOTES

Our standard is 0.25% trypsin-1 mM EDTA in PBS. However, for human ES cells, it is necessary to use lower trypsin concentrations (0.05% trypsin-1 mM EDTA) if cell viability is to be maintained for culturing after sorting.

In situ Immunofluorescence for Surface Antigens

1. Remove the medium from the cells and replace with primary antibody, diluted as appropriate in hES medium. To maintain the cultures after staining, the antibody should be sterilized by filtration using a 0.2 micron cellulose acetate filter.

2. Place the cells in the incubator for 30 minutes at 37ºC.

3. Remove the antibody–medium solution and wash three times with fresh medium.

4. Add the secondary antibody, diluted as appropriate in hES medium.

5. Place the cells in the incubator for 30 minutes at 37ºC.

6. Remove the antibody–medium solution and wash three times with PBS (with Ca^{2+} and Mg^{2+}).

7. Visualize under PBS (with Mg^{2+} and Ca^{2+}). The cells are still alive at this point, so they can be recultured if necessary. Alternatively, the cells can be fixed by treatment with a 4% PFA solution for 20 minutes, and then stored under a 50:50 solution of PBS:glycerol.

SOLUTIONS AND NOTES

The use of PBS, with Ca^{2+} and Mg^{2+} or medium, stops the cells from detaching from the tissue culture plastic.

4% PFA is 4% paraformaldehyde in PBS (without Ca^{2+} and Mg^{2+}). Heat to 65ºC to dissolve, and then

filter to remove particulates. This can be frozen at −20ºC, but should be used within two weeks. PFA is toxic, so take appropriate measures to protect yourself during preparation and usage (e.g., weigh out and make up in a fume cupboard).

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KEY WORDS

Antibody An immunoglobulin protein that specifically binds to an antigen.

Antigen A molecule that is bound in a specific manner by an antibody.

Differentiation antigen (Typically used with respect to cell surface antigens.) An antigen that is expressed by a restricted range of cells, and shows changes in expression during cell differentiation.

Epitope That part of a macromolecule that interacts specifically with a given monoclonal antibody. Typically, it comprises two or three amino acid or monosaccharide moieties, but may also depend on some aspect of the tertiary structure of the antigen.

Hybridoma Typically used to describe a hybrid cell line produced by fusing an established myeloma cell line and primary lymphocyte.

Monoclonal antibody An antibody produced by a cloned hybridoma cell line: typically, it will be a single immunoglobulin species with a defined antigen specificity.

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Chapter 48

Lineage Marking

Andras Nagy

DEFINITIONS

Mammalian development starts from a singe cell, the zygote, which is the ancestor to all the somatic and germ cells of the entire later organism. This type of developmental capacity is called "totipotency." In the first few divisions of pre-implantation development, the cells (blastomeres) retain this totipotency, but shortly before implantation, at the blastocyst stage, cells become committed to certain tissues of the later conceptus. Three cell types are clearly distinguishable at this stage: the "trophectoderm," committed to the trophoblast cells of the placenta; the "primitive endoderm," which derivatives remain extraembryonic, and will form the parietal and visceral endoderm; and the "primitive ectoderm," responsible for all cells in the embryo proper and for some internal extraembryonic membranes, such as allantois, amnion, and yolk sac mesoderm (the inner layer of this membrane). A given cell of the blastocyst is only capable of differentiating into a subset of cells of the conceptus; therefore, these cells are no longer totipotent. Instead they are referred to as multipotent (trophectoderm and primitive endoderm) and "pluripotent" (primitive ectoderm). After implantation, further subdivisions of developmental commitments occur in the embryo proper, as the embryonic ectoderm, mesoderm, and definitive endoderm form. The following organogenesis produces the final cellular diversity of an individual by differentiating highly specialized cell types ("differentiated" cells) for organ functions. The cellular diversity consists of approximately 260 distinguishable cell types in mammals, which make up an estimated total cell number of 1 trillion (10^{12}) in an adult human or 10 billion (10^{10}) in a mouse.

The progress of cell commitment to an ever-decreasing range of cell types can be viewed from the opposite direction: every cell among the 260 cell types has its own "history" that can be traced. This trace is often referred to as the "lineage" (Figure 48-1). If the traces are looked at from a given "progenitor" or a "stem cell" (the origin) in the forward direction, the set of traces is the "fate" of the cell. If the fate remains within a single type of differentiated cell, or results in two or more kinds, the origin is called "uni-," "bi-," or "multipotential," respectively. With these definitions, cell lineage and fate are practically the same phenomenon, a set of developmental traces viewed either from the differentiated product or the origin. In Figure 48-1, therefore, the fate of p1 is d1 and d2, and the fate of p2 is d1. The progenitor cell, p1, is bipotential, and p2 is unipotential. For the differentiated cell, the lineage of d2 is simple: it goes back to p1 only. The d1 lineage could be traced back to either the p1 or the p2 progenitor.

QUESTIONS TO ASK

There are two basic questions to be asked about cell lineages and cell fates:

- The lineage question: what are the traces and progenitors that lead to a certain differentiated cell type, for example, d1?

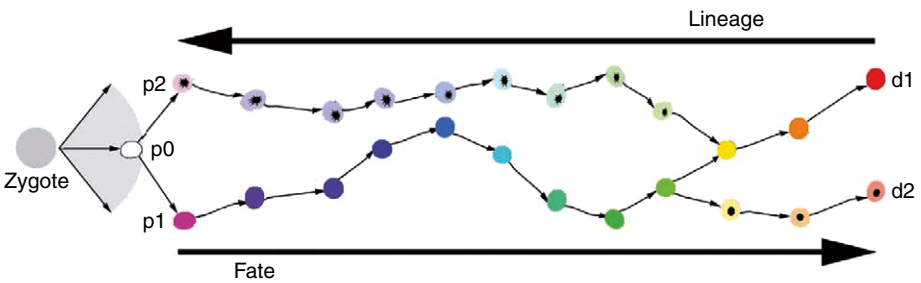


FIGURE 48-1 Relationship between cell lineage and cell fate.

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- The fate question: what are the possible differentiated products of a certain progenitor cell, such as p1?

MARKERS AND LINEAGE MARKING

As cells go through the diversification process, they correspondingly change their "gene expression profile." This profile determines the properties of the cells, and therefore their "identities." Cells of the same type express similar genes. Within this pool are some genes specific to one particular cell type. These genes are referred to as cell type-specific "markers." For example, hepatocytes, astrocytes, neurons, and cardiac myocytes can be identified by the expression of albumin, GFAP, Neu-N, and α-myosin heavy chain, respectively.

Identifying traces during development or adult regeneration is no simple task. There is often no specific marker identifying a lineage along its complete trace. Instead, a set of genes is expressed at certain segments of the lineage. For example, oligodendrocytes go through several developmental stages, such as proliferation and differentiation phases. The immature oligodendrocytes can first be identified by a surface antigen expression recognized by an antibody, A2B5. As the cells mature, they acquire another specific surface marker recognized by the antibody, called O4. Then, as they differentiate further, they start expressing myelin-specific genes, such as MBP and PLP. Typically, a temporal series of specific markers, or the lack of certain gene expression, represents a particular lineage. If these markers are known and easy to detect, they can be used to characterize the fate of a progenitor or a stem cell. However, to follow all the descendants of a given progenitor, there is a need for a unique identifier, characteristic of the progenitor under study and retained in the derivatives. Providing this identifier is "lineage marking," the subject of this chapter.

Marking with Endogenous Identifiers

It was pointed out earlier that the existence of a unique marker gene, or genes, covering an entire lineage is not common. Fortunately, however, the useful genetic differences among individuals, within or among species, can be used as identifiers in cell tissue transplantation or chimera studies. These types of lineage marking were important in the early studies of fate mapping. A great deal of knowledge was acquired using embryo transplantation chimeras between quail and chicken (see Teillet *et al.*, 1999). The nuclei of quail cells contain heterochromatin condensed into one (or sometimes two) large masses. This easily-recognizable phenomenon was used to distinguish quail from chicken cells. In the mouse, *in situ* hybridization for a specific genomic sequence of *Mus caroli* can also discriminate between these and *Mus musculus* cells in chimeric tissues. Haplotype cell-surface markers have frequently been used to study hematopoietic lineages. *In situ* hybridization for the Y-chromosome can be used as an identifier when grafting male cells into female recipients. The visualization is relatively straightforward, using Y-chromosome-specific probes on histological sections.

The unique advantage of the endogenous identifiers is that they are "built-in"; therefore, they do not require genetic or other modifications of cells. Grafting, chimera-making, and frequent requirements for bridging between genders or even species, however, limit the use of such endogenous markers. Modern transgenic methods have, in recent years, replaced these traditional approaches. Before detailing these methods, we will mention another historically important approach that provides a short-term, transient marking of a cell and its progenitors.

Marking with Dyes or Enzymes

Vital fluorescence dyes, or an enzyme with a long half-life, can mark cells in a temporary manner. Among these dyes, the most popular are DiI (a carbocyanine) and its derivatives, because of their high photostability and low toxicity. These dyes label cell membranes by inserting two long hydrocarbon chains into the lipid bilayers, resulting in an orange-red (565 nm) fluorescence emission. DiI can be used with DiO, which gives a green fluorescent color. These dyes are usually applied to cells either from an ethanol solution, or directly from the dye crystal. This simple labeling technique has frequently been used in nonmammalian model systems to successfully address lineage, potentiality, and fate questions.

Horseradish peroxidase and Rhodamine-conjugated dextran have also been used extensively for cell marking; however, these techniques are more invasive, because they have to be microinjected into the cells.

The obvious limitations to all these methods are the temporary nature of marking, and the need for free access to cells for labeling. In the mouse, the usefulness of these approaches is further limited by the requirement for *in vitro* culturing of post-implantation-stage embryos.

Marking with Exogenous Genetic Identifiers

Rapidly evolving transgene-based approaches are currently the most versatile methods of lineage marking. This fact does not mean, however, that these approaches have replaced more traditional techniques. They all have their optimal applications, and they are still valid members of the arsenal of options. There are two categories of exogenous genetic identifiers: passive and active markers.

"Passive" Exogenous Genetic Markers

Passive exogenous markers are identified on the basis of genomic insertion of a known DNA sequence into the

genome. Their detection requires techniques such as DNA *in situ* hybridization, or Southern blotting. A typical example of this category is retroviral insertion. Probing with a retroviral sequence in a genomic Southern blot detects an integration site-specific band pattern. In the case of multiple integrations, the Southern blotting can serve as a fingerprint for a group of cells derived from a single precursor or stem cell. Such an identifier can be used as a tool to measure the turnover of active stem cells that generate an entire hematopoietic compartment. Unfortunately, such an approach is not practical at a single-cell level, which restricts its application in lineage studies. Large copy number transgene insertion can also be used as a passive genetic identifier. Such a transgene creates a long enough unique and known sequence to visualize with DNA *in situ* hybridization on histological sections (see Figure 48-2A).

"Active" Exogenous Genetic Markers

Active exogenous genetic markers are based on reporter gene expression from a transgene. Several reporters are available, and have proven to be useful in lineage studies and fate mapping. Depending on the application, one may be better than the other. The most common reporters fall into two groups: enzymes, and fluorescent proteins.

Enzyme Activity-based Reporters. The β-*galactosidase* (*lacZ* gene of the *E. coli*) has had a very successful "career" as a reporter for all sorts of eukaryotic cells. The expression of the enzyme by a heterologous promoter seems to be neutral for the functioning of cells. A variety of substrates can be used for detecting β-galactosidase. The most common is an indole derivative, 5-bromo–4-chloro–3-indolyl–b-D–galactoside (X-gal), which produces a blue color as it breaks down. This simple histochemical staining works on small tissues as whole mount and on histological cryostat sections. Many useful transgenic mouse lines have been made with different specificity for LacZ expression. Of those, the most well-known is a gene trap insertion of *lacZ* into an endogenous locus named Rosa-26. This locus provides expression throughout the entire embryo, and in most adult tissues. Figure 48-2B shows an example for use of Rosa-26 transgenic and N-myc-deficient embryonic stem (ES) cells in a chimera study. The derivatives of these cells (LacZ stained blue) are not able to contribute to the chondrocyte lineage of the embryo. Since the aim of most gene trap programs is to generate *lacZ* insertions randomly in all the genes of the mouse, cell type- or lineage-specific LacZ tagging is now created in huge numbers (see Stanford *et al.*, 2001). Figure 48-3I shows an example of a LacZ-stained embryo in which the trap vector "landed" in the *Mef-2c* gene. In addition to gene trap lines, many targeted alleles that have been generated by the three high-throughput gene-targeting projects (EUCOMM, KOMP, and NorCOMM) contain

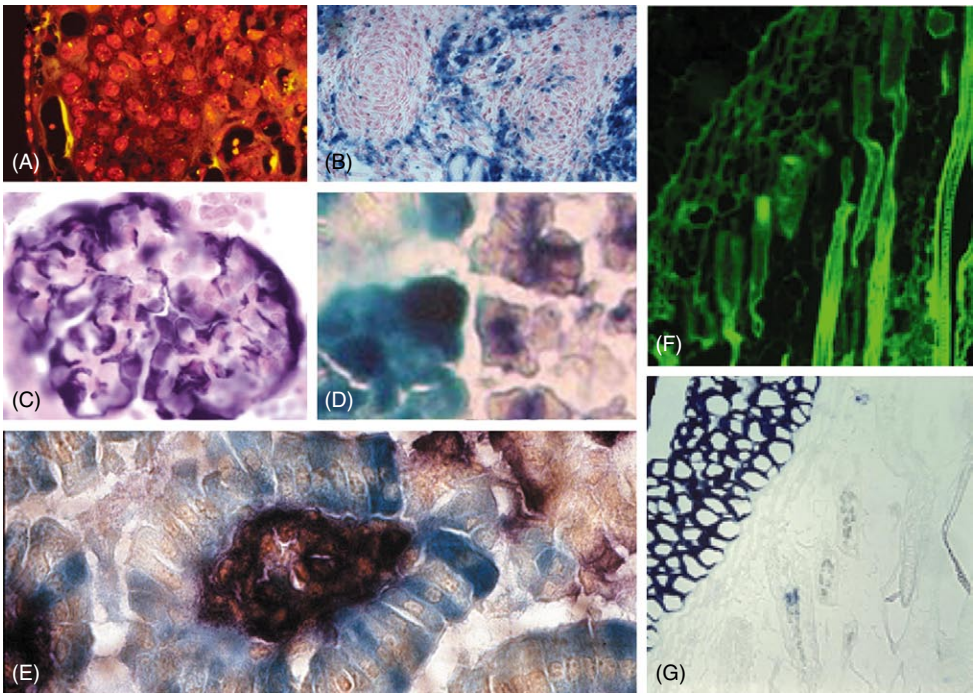


FIGURE 48-2 Lineage marking at the cellular level. (A) DNA *in situ* hybridization in trophoblast cells transgenic to a multicopy globin gene insertion. (B) Chimeric tissue where one of the components is N-myc oncogene deficient and LacZ tagged with the Rosa-26 gene trap. (C) Immunohistochemical staining for podocyte-specific expression of green fluorescent protein (GFP). (D, E) LacZ and human alkaline phosphatase double staining on mosaic intestine and pancreas. (G, F) LacZ staining and GFP visualization of an adjacent section of skin of a Z/EG, K14-Cre recombinase double transgenic animal.

a *lacZ* gene placed into the target vector in such a way that the enzyme is expressed under the regulation of the endogenous gene. Figure 48-3B presents an example in which the *lacZ* gene was knockedin to the endothelial-specific *flk-1* locus. The LacZ protein is rather tolerant to N- and C-terminal modifications. Heterologous functional domains may be added, which change the property of the enzyme, such as the intracellular localization.

Alkaline phosphatase is an essential, constitutively-expressed enzyme in cells. Similar to LacZ, a simple histochemical or whole-mount staining procedure can visualize enzyme activity in cells. Endogenous alkaline phosphatases can easily be inactivated by a brief heat treatment (at 70ºC for 15 minutes). However, the alkaline phosphatase expressed by the human placenta (hPLAP) is unique, in that it is not affected by such a heat treatment. This unique property has been utilized for detection of hPLAP activity when expressed from a transgene. Since heat treatment can be applied both to whole-mount and cryostat sections, hPLAP has become a convenient histological marker, which allows simple detection–recognition of enzyme-containing cells. Since hPLAP is efficiently transported into axons, it is an ideal reporter if nerve visualization is required. Figure 48-3C shows sporadic hPLAP+ cells in a 10.5-day postcoitus (dpc) embryo, and Figures 48-2D and E show double staining for LacZ and hPLAP on a cryostat section of intestine and pancreas of a mosaic newborn (for more details, see the next sections of this chapter). However, compared to LacZ, hPLAP stain penetration to whole-mount tissue is slightly more limited, and the quality of histological sections falls short of excellence, because of the heat treatment of the tissue.

Fluorescent Proteins. The green fluorescent protein (GFP) isolated from the jellyfish (*Aequorea victoria*) has recently joined the group of reporters used to trace lineages, and determine cell fate in mouse and other model organisms. Several mutant derivatives have been developed providing different levels of stability and intensity, and a large spectrum of light emission from blue to red. The gene that encodes for fluorescent protein (FP) can be applied in heterologous-transient or -stable integrant transgenic expression settings or its mRNA for transient production of the protein. This reporter is unique, because its visualization does not require cell fixation or staining. Instead, live specimens can be observed, and cells expressing FP can be followed in their dynamic behavior. However, not even FPs could overcome the main obstacle in studying mammalian development—that the embryo

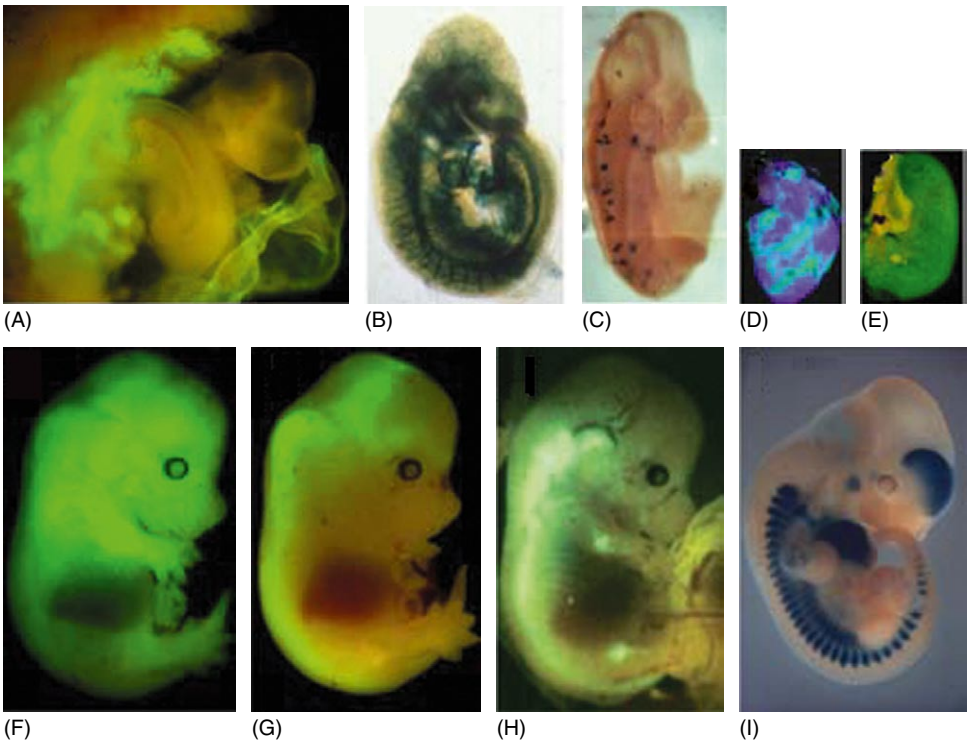


FIGURE 48-3 Whole-mount embryos and organs with lineage-marked cells. (A) ES cell-derived embryo in the uterus. The trophoblast of the placenta and the yolk sac endoderm is GFP transgenic tetraploid embryo-derived. (B) Whole-mount LacZ stained embryo in which the *lacZ* gene is inserted into the flk-1, endothelial cell specifically expressed receptor kinase. (C) Z/AP and Cre recombinase double transgenic embryo with sporadic human placental alkaline phosphatase activation. (D) Heart of the chimeric embryo between cyan fluorescent protein expressor ES cells and GFP transgenic embryo. Z/EG and Cre recombinase double transgenic embryos with (E) GFP podocyte-specific; (F) complete; (G) differentiated neuron-specific; and (H) chondrocyte-specific activation. (I) LacZ-stained embryo derived from a Mef-2c gene trap ES cell line.

needs an *intra-utero* environment. *Ex vivo* organ culture systems are gaining importance to overcome this limitation; however, it is not yet possible to follow the very early post-implantation time (4.5–6.5dpc) or beyond midgestation (11.5dpc) *in vitro*.

Establishing Overall Expressor Transgenic Mouse Lines. When cell grafting is the approach to studying cell fate, it is vital that all derivatives of the graft be identifiable. For this purpose, the reporter has to be expressed in all the cells of the graft. For over 17 years now, the Rosa-26 gene trap line has been the best LacZ expressor reporter in the mouse. Second on the "popularity list" is a LacZ line that has the transgene integration on the X-chromosome. This line has been used successfully in several studies to address cell-fate and lineage determination questions during gastrulation.

Establishing a new overall expressor transgenic mouse line is a challenging task. Even the most promising promoter gives unstable, mosaic, or restricted expression from most insertion sites in the genome. A random insertion transgenic approach based on pronuclear DNA injection requires a very large number of founder animals to be established and tested for expression. For this reason, ES cell-mediated transgenesis may be a better alternative. If the same constructs are introduced into ES cells, rather than directly in the embryos, hundreds of transgenic clones can be isolated in a short time. These clones, which all represent a unique genomic integration site of the transgene, can be screened for the expression level and pattern of the reporter, both in undifferentiated ES cells, and in *in vitro* differentiation assays. If only ES cell lines with overall expression are used to generate mice, the chance of obtaining satisfying reporter expression is high.

ES cells may also provide control of the transgene insertion site. The most common method is targeted integration of a transgene (reporter) into a well-characterized locus, for example, Rosa-26, or the X-chromosome-linked HPRT. Recombinase-mediated cassette exchange is an alternative that could be used to eliminate the uncertainty of transgene expression caused by random genomic insertion. Three recombinase–integrase systems are available for such an approach; the Cre, Flip (Flp), and PhiC31. By using any of these, a "docking" site can be prepared and characterized for expression permissiveness. The transgene is then equipped with a recognition site or sites of the recombinase, and introduced into the cells together with the enzyme. Site-specific genomic integration of the transgene can thus be achieved at a high efficiency. Further details about this approach can be found in Chapter 9 in Nagy *et al.*, 2003.

The Chimera: A Tool for Fate Mapping

The possibility of combining two or more embryos in one chimera contributed tremendously to the understanding of lineage determination and cell fate in mouse development (for a review, see Nagy *et al.*, 2001). Identifiers described previously are essential to detecting and characterizing the contribution of a chimeric component to a lineage, cell type, or organ. Injection of cells isolated from the pre-implantation embryo into a blastocyst, and characterization of the allocation of derivatives of each compartment in later stages revealed the first specification events. Chimeras (Figure 48-3D) have also been very informative in addressing basic organization questions—for example, whether complex structures are derived from single progenitors (clonality in development).

The use of ES cells and tetraploid embryos has expanded the horizon of questions that could be addressed. ES cells are not able to contribute to the trophectoderm and primitive endoderm lineages of the developing chimeras. On the other hand, cells derived from tetraploid embryos are excluded from the primitive ectoderm lineage when they have to compete with diploid embryo or ES cell derivatives. For chimera production, one can choose any two of the following three sources as components: diploid embryo; tetraploid embryo; and ES cells. In addition, two diploid embryos can be chosen, increasing the number of possible combinations to four. Depending on the actual combination, different lineage allocation can be obtained for the selected components (see Nagy and Rossant, 2001) (see also Figure 48-4). The most extreme separation occurs when tetraploid embryos are combined with ES cells; practically no chimeric lineages are generated. The cells in the resulting embryo are either tetraploid-derived (trophoblast lineage of the placenta, visceral, and parietal endoderm) or ES cell-derived (amnion, allantois, embryonic mesoderm component of the placenta and yolk sac, umbilical cord, and embryo proper) (Figure 48-3A). In a broader sense, the lineage restrictions in chimeras could be considered lineage marking.

Cell and Tissue Grafting

The classical chimera production restricts the components to the sources discussed in the previous paragraph, and the timing to the pre-implantation stages. Later stage cell mixing is also possible but demanding, and the possible applications are more limited. Small-tissue transplantation can be performed between two post-implantation stage embryos. If the graft is tagged with a unique identifier, the fate of these cells can be followed during development of the recipient. This technique has been in practice for almost 20 years. Developmental trajectories, as they are depicted in Figure 48-1, are dynamic processes, not only at the level of gene expression, but also that of cell allocations in the developing embryo. These two levels interact: allocation can induce gene expression changes; and gene expression changes can influence cell allocations. Knowing the complexity of mammalian gastrulation, one

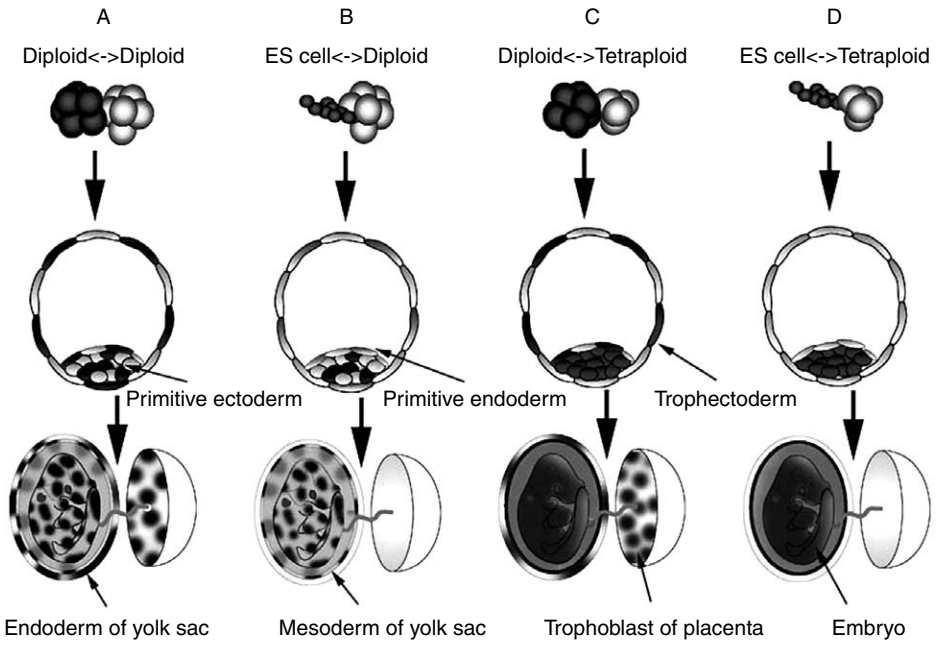


FIGURE 48-4 Diagram of lineage contributions in different kinds of aggregation chimeras. Solid colors indicate nonchimeric tissues, and stripes indicate chimeric tissues.

can easily imagine the heroic effort of creating a map of cell movements in the early post-implantation embryo using mostly LacZ-marked tissue grafting, and still pictures showing the allocation of the graft derivatives.

Further limitations associated with this approach derive from our inability to successfully place the post-implantation-stage embryo back into the uterine environment where it could develop further. *In vitro* post-implantation embryo culture sets a limitation on the "developmental stage window" of isolation from 6.5dpc to 10.5dpc, and on survival for a period of only one to three days.

Using Genetic Switches

Grafting identifier-tagged tissues from one embryo to another creates a small population of progenitor cells whose derivatives can easily be recognized at later stages. The experimental achievement of such a scenario is rather invasive, and is hampered by the limitations pointed out in the previous paragraph. State-of-the-art tools, however, can be used to achieve a similar situation with much less invasiveness. The key element is the Cre site-specific recombinase, which recombines DNA between two consensus loxP sites. If two loxP sites are similarly oriented in the same DNA strand, the result of such a recombination is the excision of the intervening sequence. This property allows the design of the Cre excision-conditional reporter *Rosa26R*, a knockin transgenic mouse line that uses the widespread expression from the Rosa26 locus, where now the LacZ expression is conditional to Cre excision.

A similar reporter line has been developed for the Flp recombinase system. The *Z/EG* and Z/Red transgenes are more sophisticated "switch" reporters, with the following features: a strong overall expressor promoter is driving a *lacZ-neomycin* resistance fusion gene (b*geo*) followed by three polyadenylation (pA) sites. The b*geo* and the 3xpAs are flanked by loxP sites, and a coding region of an enhanced green fluorescent protein (EGFP) or dsRed-MST with its own pA follows this flanked region. These transgenes were inserted as a single copy into a single site of the mouse genome leading to the expression of the β*geo* only. However, EGFP or dsRed-MST expression can be activated by Cre recombinase; Cre activity removes β *geo*+3xpA, and moves the EGFP or dsRed-MST coding region under the transcriptional control of the promoter (Figure 48-3E–H). The EGFP/dsRed-MST activation is, therefore, dependent on the presence and action of the Cre recombinase. Z/AP, another reporter line, can be used in the same way as Z/EG or Z/Red, except that the second reporter is hPLAP instead of EGFP.

Cre recombinase has had a great career in mouse genetics (see Nagy, 2000). Hundreds of transgenic mouse lines have been produced, expressing this phage enzyme with different spatial and temporal control. (http://nagy.mshri.on.ca/cre). Double transgenic combination of a Cre transgene and the Z/EG (or Z/AP, Z/RED) reporter turns on EGFP (or hPLAP, dsRed-MST) expression, not only in the actual Cre expressor cells, but also in any cell that had an ancestor expressing the recombinase during the course of development. Therefore, the EGFP+ cells in these double transgenic embryos or animals are the union of all the cells expressing the recombinase, and those that had a Cre recombinase expressor ancestor (Figure 48-3E–H). It is easy to recognize that an efficient Cre recombinase excision is not always advantageous in this situation, since the resulting set of EGFP+ (hPLAP+, dsRed-MST)

cells could be too large, obscuring the recognition of fates derived from a single Cre+ progenitor. A less efficient Cre recombinase is preferable here. If the excision (activation of the second reporter) only occurs sporadically, the allocation of the descendent cells may still reflect the clonality (Figure 48-3C), and reveal the potential of the progenitor. Low-frequency activation of the Cre recombinase can be achieved by the use of either the tamoxifen- or the tetracycline-inducible Cre recombinase systems. Proper titering of the inducer can create informative excision–activation frequency in progenitors. In addition, withdrawal of the inducer may be used to create an upper developmental limit of the excision–activation of the reporter.

The induction of low-frequency or controlled activation of the conditional reporter is also possible by direct injection of the recombinase, or its mRNA, into cells of developing embryos.

FUTURE DIRECTIONS

It is difficult to predict what the distant future will bring into this exciting area. In the near future, however, efforts will certainly lead to novel features that will provide the means to follow the behavior of cells. New imaging techniques will allow the recording of the dynamic property of cells, such as movements, speed, direction, interactions with other cells in *in vitro* cultures, or formation of specific embryonic structures in *ex vivo* cultures of early post-implantation embryos. The study of later development may require slice cultures for recording cellular events, such as the birth of neurons from radial glia cells. Another technical development, ultrasound-guided cell grafting into embryos is expected to have an effect on lineage studies and fate mapping. It is becoming possible to graft reporter-tagged cells into post-implantation-stage embryos while *in utero*, and to recover the embryos at a later stage for studying the derivatives of the graft.

Remembering the enormous effort behind generating the complete map of lineage development in *C. elegans*—which consists of less than 1000 cells—the task of untangling the same system in the mammalian embryo may seem impossible. This conclusion comes not only from the dramatic increase in cell number, but also from the increased plasticity of higher organisms. Plasticity creates a stochastic component in differentiation and cell determination processes. Therefore, the lineage studies and fate mapping have to take this uncertainty component into account.

Our ever-deepening understanding of self-renewal and differentiation has recently led us to the ability to revert the developmental processes. Somatic, terminally-differentiated cells can be reprogrammed to an early embryonic pluripotent state by force expression of just a few transcription factors, including Oct4 and Sox2. This exciting new area of induced pluripotent stem (iPS) cells opens a new dimension of lineage and fate mapping studies. Finding out the nature of "backwards" differentiation has enormous potential in regenerative medicine, a potential future cell-based cure for currently devastating degenerative diseases.

Nevertheless, the increasing understanding of lineage determination and differentiation will place us in a good position to control these processes, both *in vivo* and *in vitro*. ES cells have been playing an important role in this process. The acquired knowledge, in return, promotes the development of technologies aiming for controlled *in vitro* differentiation of ES cells into therapeutically useful cell types.

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KEY WORDS

Cell fate Future possible course of differentiation of a given cell.

Cell lineage Set of courses of differentiation that leads to a given cell type.

Differentiation Changes in biological, physical, and/or chemical cell properties associated with the development of final cell types of an organism.

Proliferation Cell division without a change in cell properties.

Pluripotent Capability of a cell to develop into many, but not all, the cell types of an organism.

Totipotency Capability of a cell to develop into all cell types of an organism.

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Chapter 49

Genomic Reprogramming

M. Azim Surani

INTRODUCTION

Most cells contain the same set of genes, and yet they are extremely diverse in appearance. They are all derived from a totipotent zygote generated after fertilization of an oocyte. William Harvey, in 1651, was the first to propose that "everything comes from an egg," which also encapsulated the concept of "epigenesis," or the gradual emergence of the embryo and fetus from an egg. Nearly 300 years later, Conrad Waddington elaborated on epigenesis in his famous sketch depicting the "epigenetic landscape." Developmental biologists, beginning with Jacques Loeb and Hans Spemann, also began to consider whether all nuclei could be reprogrammed and become totipotent in nuclear transfer experiments. From many subsequent experiments, it became clear that the maternally-inherited factors contained within the oocyte also have the extraordinary property to restore totipotency to a differentiated somatic nucleus when transplanted into it. The components within the oocyte must have the property to alter the somatic nucleus so that it can recapitulate the entire developmental program, and thus give rise to an exact genetic copy or clone of the individual who donated the transplanted nucleus. This transformation of differentiated cell to a totipotent state is probably the most widely understood meaning of genomic reprogramming. However, it is important to note that extensive epigenetic reprogramming of the genome also occurs in the germline and during early development, which is essential for generating the totipotent zygote, and for creating the pluripotent epiblast cells from which both germ cells and somatic cells are subsequently derived.

Specification of diverse cell types from pluripotent cells is determined by the expression of a precise set of genes, while the rest are repressed. These newly-acquired cell fates are propagated by heritable epigenetic mechanisms through modifications of chromatin and by DNA methylation. These epigenetic modifications, although they are heritable, are also reversible and can be erased, which is why it is possible to change the phenotypic characteristics of cells and restore totipotency to somatic nuclei under specific conditions (see Figure 49-1). To understand the mechanisms of reprogramming, it is important to know the nature of chromatin modifications and the mechanisms that can reverse or erase the existing modifications, and also how new modifications are imposed. Because these reprogramming factors normally play a significant role during early development, it is important to determine their role in this context, and how these factors act on somatic nuclei during restoration of totipotency or pluripotency.

GENOMIC REPROGRAMMING IN GERM CELLS

Germ cells provide the enduring link between generations, and for this reason, this lineage exhibits many unique properties, including the extensive epigenetic reprogramming of the genome prior to gametogenesis. This reprogramming is crucial for generating viable and functional gametes, which in turn generate a totipotent zygote. Primordial germ cells (PGCs) are among the first cells to

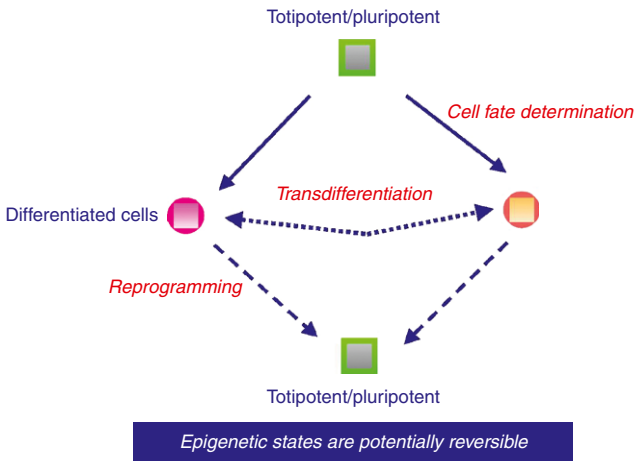


FIGURE 49-1 Genomic reprogramming involves heritable but reversible epigenetic modifications.

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undergo specification from pluripotent epiblast cells, when the distinction between germ cells and soma is established. PGCs as precursors of sperm and oocyte are highly-specialized cells, and are the only cells that can undergo meiosis. However, PGCs retain expression of some markers of pluripotency, such as *Oct4*. It is also possible to derive pluripotent embryonic germ cells (EG) from PGCs. In this context, it is interesting to determine both how PGC specification occurs, and how these cells undergo dedifferentiation to pluripotent EG cells, which may provide some insights into genomic reprogramming.

Stem Cell Model for the Specification of Germ Cells in Mammals

There are two key mechanisms for the specification of germ cells. The first involves inheritance of preformed germ plasm, which is found in *Drosophila* and *C. elegans*. In mammals, germ cell specification occurs according to the stem cell model, where germ cells are derived from pluripotent epiblast cells in response to signaling molecules from the extraembryonic ectoderm. BMP4 and BMP8b are among the key signaling molecules in conferring germ cell competence on pluripotent epiblast cells in mice, starting at E6.5 (Figure 49-2), an event that is detected by the expression of *fragilis*, a transmembrane protein. These germ cell competent cells are initially destined for a mesoderm somatic cell fate, as they show expression of *Brachyury* and some region-specific *Hox* genes as they migrate toward the posterior proximal region. However, at around E7.25, cells that ultimately acquire a germ cell fate switch off the somatic program through repression of a number of genes, which continue to be expressed in the neighboring somatic cells. Cells that acquire germ cell fate continue to show expression of markers of pluripotency, including *Oct4*. A unique marker of germ cells at this time is *stella*, which is first detected in the 45–50 founder germ cells at E7.5. Thus, diversification between germ cell and somatic cell fate among neighboring cells occurs over approximately 6–10 hours between E7.25–E7.5, with the repression of somatic cell programming being one of the major events during germ cell specification.

From Stem Cells to Germ Cells

Recent studies show that it is also possible to derive PGCs and gametes from pluripotent stem cells. In one study, ES cells with *gcOct4-GFP* reporter that drives expression specifically in germ cells were generated. These ES cells with the reporter were allowed to undergo differentiation when cells with GFP were detected. These cells showed expression of a variety of germ cell-specific markers. Further cultures following cell sorting eventually produced oocyte-like cells that underwent development to form blastocyst-like structures. The latter shows that mouse ES cells can, under these conditions, differentiate into oocytes and, subsequently, blastocysts. With further detailed characterization of germ cells and gametes, such an *in vitro* system may be useful for investigations concerning specific aspects of germ cell development.

Another study has similarly been carried out to generate spermatogenic cells from pluripotent ES cells. In this case, the endogenous mouse homolog of *Vasa*, *Mvh* was used to knockin the reporters, *LacZ* and *GFP*. In this study, germ cells were generated in embryoid bodies, which were detected through the expression of MVH-GFP. This process was greatly enhanced by the exposure of ES cells to BMP4. These MVH-GFP cells were aggregated with

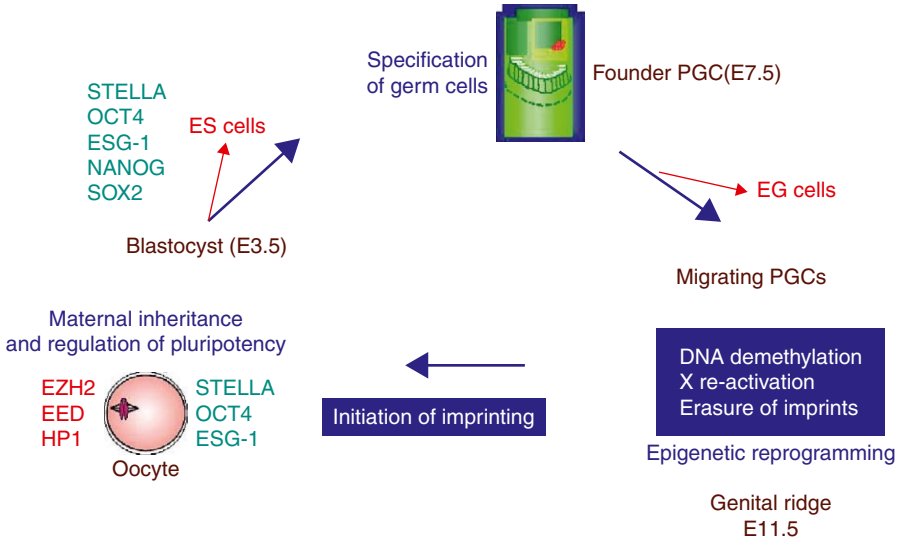


FIGURE 49-2 Genomic reprogramming in the oocyte, early embryos, and the germline. The origins of pluripotent embryonic stem cells (ES) and embryonic germ cells (EG) are indicated.

E12.5–13.5 male gonadal cells when germ cells within these aggregates developed into elongated spermatids.

These studies show that it may be possible to generate an efficient *in vitro* system to derive germ cells from pluripotent ES cells. Such a system would be useful to study the mechanism of PGC specification *in vitro*, as well as other aspects, including the formation of gametes, and aspects of epigenetic reprogramming of the genome. Derivation of germ cells from human ES cells would be particularly useful for studies on this lineage. Furthermore, derivation of human oocytes from ES cells would greatly add to this scarce resource, thus providing opportunities for fundamental studies on somatic cell reprogramming, and for the subsequent derivation of stem cell lines from human somatic cells for investigations of specific mutations and diseases.

From Germ Cells to Stem Cells

Embryonal carcinoma cells (EC) that are derived from PGCs *in vivo* were the first pluripotent stem cells to be identified, and several loci have been identified that have a critical role in this process. At the same time, derivation of pluripotent cells from PGCs has also been achieved *in vitro*. This conversion of germ cells into EG cells occurs in the presence of leukemia inhibitory factor (LIF), basic fibroblast growth factor (FGF2), and the Kit ligand (KL). The precise mechanism for conversion of the highly-specialized germ cells into pluripotent stem cells is largely unknown. Further investigations would provide insights into dedifferentiation of cells, and on the mechanism of genomic reprogramming.

Epigenetic Reprogramming in Germ Cells

One of the properties of germ cells, of particular interest, is the epigenetic reprogramming of the genome. This event occurs when PGCs enter into the developing gonads (Figure 49-2), when there is extensive erasure of epigenetic modifications, including erasure of genomic imprints and reactivation of the inactive X-chromosome. New parental imprints are initiated later during gametogenesis, particularly oogenesis, and these modifications that are heritable after fertilization dictate parent-of-origin dependent gene expression.

As germ cells proliferate after the formation of the founder population at E7.5, they start to migrate to the developing gonads. At this stage, germ cells, as well as somatic cells, contain epigenetic marks associated with imprinted genes. During their migration, female germ cells also show inactivation of one X-chromosome. Upon the entry of germ cells into developing gonads at E10.5–E11.5, a major epigenetic reprogramming event occurs, which includes reactivation of the inactive X-chromosome, and the erasure of epigenetic marks associated with imprinted genes. Indeed, there appears to be genomewide DNA demethylation of the genome at this time. This genomic reprogramming event occurs relatively rapidly, and it is completed by E12.5.

The mechanism involved in the erasure of epigenetic modifications in the germline may provide insights into the erasure of epigenetic modifications from somatic nuclei that occur after transplantation into oocytes, which restores totipotency. If this is the case, similar factors may be transcribed during oocyte maturation at the germinal vesicle stage and translated, to be stored in oocytes as maternally-inherited factors. The onset of genomic reprogramming in PGCs in the gonad may be triggered by a signal from somatic cells, or in response to a developmental timer, such as the number of cell divisions in PGCs since the establishment of the founder population of PGCs. Because the gonads at E11.5 are bipotential, it is possible that the signal from somatic cells, if it exists, should be the same in both male and female embryos. In this case, it would be of interest to discover the nature of the signal and determine how such an external cue can trigger extensive genomic reprogramming. However, there is also support for the alternative developmental timer model, since EG cells show erasure of imprints even when they are derived from PGCs where this process has not yet commenced. It is possible that erasure is initiated when germ cells complete a critical number of cell cycles, although, as these cells are cultured in a complex medium, a role for an environmental cue cannot be entirely discounted. Whatever the case may be, it is important to note that EG cells themselves have the property to induce erasure of epigenetic modifications from somatic nuclei (see below).

Genomic Reprogramming in Oocytes

Resumption of oocyte growth is accompanied by further epigenetic reprogramming events, particularly the initiation of genomic imprints. The majority of the epigenetic marks associated with imprinting are introduced during oocyte growth, although some genes acquire paternal-specific imprints in the male germline. These epigenetic marks are eventually detected as DNA methylation of specific *cis*-control elements. Some of the marks, for example, in the *Igf2r* locus, ensure that the gene will be active only when maternally-inherited, while others such as *Peg3* will be silent in the female genome. *Dnmt3l* is a key gene involved in the initiation of parental imprints, which acts together with the *de novo* DNA methylase enzyme, Dnmt3a. A mutation in the *Dnmt3l* gene does not disrupt development or maturation of the oocyte, except that these oocytes do not carry appropriate maternal imprints or epigenetic marks. Following their fertilization, the resulting embryos are unable to develop normally, and they die shortly after implantation. Other genes, such as *H19*,

undergo DNA methylation in the paternal germline, and this gene is repressed in the paternal genome. This topic is discussed comprehensively elsewhere.

Maternal Inheritance and Reprogramming of Parental Genomes

As in other organisms, mouse oocytes contain a number of maternally-inherited proteins and messages (Figure 49-2). In mammals, maternally-inherited factors are essential for totipotency and pluripotency, such as Oct4, Esg1, and Stella, although there is no Nanog. Maternal inheritance of Stella is apparently necessary for normal pre-implantation development. The oocytes also contain epigenetic modifiers, including the *Polycomb* group proteins, Ezh2 and eed, as well as the heterochromatin factor, HP1. These factors are essential for regulating early development, and for generating the pluripotent epiblast and trophectoderm cells of blastocysts. The oocyte is also likely to inherit some key chromatin remodeling factors.

In mammals, the parental genomes exhibit epigenetic asymmetry in the zygote as a result of imprinting, which confers functional differences between parental genomes. At fertilization, the maternal genome apparently has high methylated lysine 9 histone H3 (H3meK9). Immediately after fertilization, the hetrochromatin protein HP1b binds preferentially to the maternal genome. The Polycomb proteins, Ezh2 and eed, also bind preferentially to the maternal genome. While this takes place, the paternal genome that has relatively low levels of H3meK9 shows genome-wide DNA demethylation, thus enhancing the epigenetic differences between the parental genomes. Ezh2 has the conserved suvar E2 trithorax (SET) domain with histone methylase activity for methylation of histone H3-lysine 27/lysine 9 (H3meK27/9). The maternal inheritance of Ezh2 *per se* is apparently important, since depletion of this factor from oocytes results in development of very small neonates, presumably because of an effect on placental development. This seems likely because the neonates eventually grow and acquire normal size, indicating a placental functional deficiency during development. Whether this is due to an effect on imprinted genes remains to be determined. These experiments show that factors present within the oocyte have the potential to exert a variety of epigenetic effects on development. Somatic nuclei transplanted into oocytes would be affected by the activities of these factors during reprogramming, but the variable expression of genes associated with totipotency and of imprinted genes argues that appropriate epigenetic reprogramming of the genome may not be accomplished in every case (see below).

Factors involved in chromatin remodeling are also likely to be important for early development and genomic reprogramming, as they regulate accessibility to DNA (Tada and Tada, 2001). The SWI/SNF-like complexes consist of at least two ATPase subunits, BRG1 and BRM. *Brg1* is important during pre-implantation development, as loss of function is lethal during this phase. It is also known that mutation in *ATRX*, a member of the SNF2 helicase/ATPase family, has an effect on DNA methylation of highly-repeated sequences. Mutation in *Lsh* similarly results in substantial demethylation of the genome. Lsh is related to the SNF2 subfamily; most members of the SNF2 family of proteins appear to have the capacity to alter chromatin structure. The activity of the nucleosome-dependent ATPase, ISWI, may be used in chromatin remodeling in nuclear reprogramming of somatic nuclei; if so, it is likely to be present in the oocyte, and would have a role in the zygote. One of the earliest changes observed following fertilization (or indeed after transplantation of the somatic nucleus) is the apparent increase in the size of the nucleus. This morphological change may be in response to chromatin remodeling factors belonging to the ISWI complexes. This activity may be necessary for the initial unwinding of the chromosomes, to facilitate epigenetic modifications of the chromatin.

Reprogramming During Early Development

Epigenetic reprogramming of the embryonic genome continues throughout pre-implantation development, as judged by the continual changes in histone modifications and a decline in the genomic levels of DNA methylation. During pre-implantation development, both pluripotent epiblast and differentiated trophectoderm cells are formed. There are differences in the epigenetic reprogramming in these two tissues. For example, there is preferential paternal X-inactivation in the trophectoderm, a process in which the *Polycomb* group proteins, Ezh2/eed complex, have a significant role. The cells of the late morula, which are positive for the expression of *Nanog*, and destined to form the inner cell mass, cease to show Ezh2/eed accumulation at Xi, as seen with the paternal X-chromosome in the trophectoderm. Ezh2 is also detected in the inner cell mass, which may account for the presence of the overall H3meK27 staining of epiblast cells, which are positive for Oct4 expression.

It appears that histone modifications, such as H3meK27, may have a role in the maintenance of epigenetic plasticity of the pluripotent epiblast cells, since the loss of function of Ezh2 is early-embryonic-lethal, and it is not possible to derive pluripotent ES cells from blastocyst that are null for Ezh2. These experiments show the importance of appropriate epigenetic reprogramming of the genome for early development, and for generating pluripotent epiblast cells that are the precursors of both somatic and germ cell lineages. As we learn more about nuclear reprogramming events that occur normally in germ cells, oocytes, and early development, these studies are

likely to be used to identify key candidates for genomic reprogramming.

REPROGRAMMING SOMATIC NUCLEI

Nuclear Transplantation

Epigenetic reprogramming of somatic nuclei transplanted into oocytes must require erasure and initiation of appropriate epigenetic modifications compatible for development. This subject has already been reviewed extensively elsewhere. At least some of the key reprogramming events may be faulty, to account for the very low success rate, since somatic nuclei undergo variable reprogramming resulting in a wide variety of phenotypes. The effects of aberrant reprogramming are apparent, particularly soon after implantation and during post-implantation development. Both the embryo and extraembryonic tissue seem to be affected. Some epigenetic marks associated with imprinted genes are erased, resulting in aberrant expression of these genes. It seems likely, therefore, that a large number of genes fail to show appropriate temporal and spatial patterns of expression. Further studies on the mechanisms of genomic reprogramming during normal development and following nuclear transplantation are necessary to assess the reasons for faulty reprogramming of somatic nuclei.

Reprogramming in Embryonic Stem-somatic and Embryonic Germ-somatic Cell Hybrids

Somatic nuclear reprogramming has also been demonstrated in hybrid cells between pluripotent ES/EG and somatic cells, which also restores pluripotency in somatic nuclei. These studies indicate that not only the oocytes, but also pluripotent ES/EG cells must contain appropriate factors to reprogram the somatic nucleus. Reprogramming of somatic nuclei in ES/G-somatic cell hybrids is, however, relatively less complex compared to its transplantation into oocytes. This is because the somatic nucleus in the oocyte has to be reprogrammed to recapitulate the entire program of early development to the blastocyst stage. It is important to note that this donor somatic nucleus has to be reprogrammed to generate pluripotent epiblast cells, as well as the highly-differentiated trophectoderm cells. The latter should be viewed as a trans-differentiation event, because somatic nuclei of diverse origin must direct differentiation of highly-specialized trophectoderm cells after only a few cleavage divisions. Indeed, in some respects, this trans-differentiation event is more striking as a reprogramming event. By comparison, reprogramming of somatic nuclei in ES/EG–somatic cell hybrids is less complex, as there is restoration of pluripotency without the necessity to recapitulate early events of development.

Although EG and ES cells on the whole have similar effects on somatic nuclei, there is at least one critical difference between them. Using EG–thymocyte hybrid cells, it was shown that the somatic nucleus underwent extensive reprogramming resulting in the erasure of DNA methylation associated with imprinted genes, and the inactive X-chromosome was reactivated. The somatic nucleus also acquired pluripotency, as judged by the activation of the *Oct4* gene, and the hybrid cells could differentiate into all three germ layers in chimeras. This study shows that EG cells, apart from conferring pluripotency to somatic nucleus, retained a key property found only in germ cells, which is the ability to erase parental imprints and, indeed, induce genome-wide DNA demethylation. Experiments using ES–thymocyte hybrid cells gave similar results, including the restoration of pluripotency to somatic nuclei, as shown by the activation of the *Oct4–GFP* reporter gene, and for the ability of these cells to differentiate into a variety of cell types. Unlike EG cells, however, ES cells do not cause erasure of imprints from somatic nuclei. Furthermore, in ES–EG hybrids, EG cells can induce erasure of imprints from ES cells, which shows that EG cells have dominant activity for the erasure of imprints and DNA demethylation. However, from these studies it is clear that DNA demethylation activity, at least for the erasure of imprints present in EG cells, is not essential for restoring pluripotency to somatic cells. It is possible to use this system to design cell-based assays in search of key reprogramming factors.

The ability of ES/EG cells to restore pluripotency in somatic nuclei is significant because it also opens up possibilities to identify the molecules involved in reprogramming somatic nuclei. Such studies are difficult with mammalian oocytes, partly because they are small compared to amphibian oocytes, and it is difficult to collect large numbers of them. More importantly, as discussed earlier, oocytes are complex cells containing factors essential for pluripotency, as well as for the early development and differentiation of trophectoderm cells. By contrast, pluripotent ES/EG cells are relatively less complex, and, more importantly, they can be grown indefinitely *in vitro*. Thus, they can provide a considerable source of material for analysis. For example, it is possible to use nuclear extracts from ES/EG cells to examine reprogramming of somatic nuclei, as described in one experimental approach. The availability of relatively large amounts of nuclear extracts from ES/EG cells also makes it possible to undertake biochemical studies to identify the key reprogramming factors.

CONCLUSIONS

The evidence from studies on early mammalian development shows that there is dynamic and extensive reprogramming of

the genome in the oocyte, zygote, and germ cells. Pluripotent stem cells also appear to show considerable potential for genomic reprogramming, and while there are differences between ES and EG cells, both of them can restore pluripotency to somatic nuclei. There is maternal inheritance of factors for pluripotency, epigenetic modifications, and chromatin remodeling in the oocyte. Reprogramming in the oocyte is relatively complex, since the parental pronuclei exhibit epigenetic asymmetry in the zygote. The paternal genome becomes rapidly demethylated after fertilization, but the maternal genome does not, at least in part, because of the differential histone modifications, such as the preferential H3meK9 of the maternal genome, but not of the paternal genome, and the preferential binding of HP1b and Ezh2/eed proteins to it. It is possible that epigenetic reprogramming of somatic nuclei may be affected by their original epigenetic state.

The phenomenon of reprogramming somatic nuclei is now well-established in mammals, both by nuclear transplantation studies and in heterokaryons, but the mechanisms and the key molecules involved are yet unknown. It is reasonable to assume that some of the factors involved in reprogramming the genome in the germline are also present in the oocyte. It is also possible that some of the basic reprogramming factors present in pluripotent stem cells are also present in the oocyte.

A likely sequence of events for converting a somatic nucleus to a pluripotent nucleus may first require chromatin-remodeling activity. Many of these complexes are known to exist in mammals, but at this stage it is not known precisely which are important for reprogramming of somatic nuclei. This may be followed by changes in histone modifications compatible with pluripotency. What these changes are has yet to be fully determined, together with the identity of the histone modifiers.

As ES/EG cells apparently have the capacity for reprogramming somatic nuclei to pluripotency, they may be used for identifying key molecules necessary for genomic reprogramming through cell-based assays, combined with appropriate biochemical and cellular analyses.

KEY WORDS

Chromatin DNA and associated proteins that create appropriate conditions for the expression or repression of genes during development and differentiation of cells.

Epigenesis The formation of entirely new structures during development of the embryo.

Epigenetic The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences.

Genomic reprogramming The process that alters functions and properties of a cell through reversible modifications of DNA or associated proteins, without affecting the DNA sequence itself.

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Chapter 50

Isolation and Characterization of Hematopoietic Stem Cells

Gerald J. Spangrude and William B. Slayton

INTRODUCTION

Central to the development of methods for stem cell isolation is the availability of quantitative techniques for assessment of stem cell function. In this regard, any approach to identification and isolation of adult stem cells can be no better than the assay used to detect function. This is a critical issue with blood stem cells, because the robust nature of hematopoiesis requires massive expansion of very few stem cells to provide a continual source of replacements for mature cells that die every day. The degree of expansion that occurs from stem cell to progenitor cell to mature cell is vast enough to prevent absolute distinction between the most primitive stem cells and the differentiating progeny of these cells. Methods for hematopoietic stem cell (HSC) isolation described here include approaches that minimize co-isolation of non-stem cells, while also providing techniques to isolate populations of progenitor cells possessing remarkable proliferative potential in the absence of stem cell activity. Comparison of primitive hematopoietic stem cells with early progenitor cells will provide interesting insights into the early stages of hematopoietic development.

ISOLATION OF HEMATOPOIETIC STEM CELLS FROM MICE

Historical Perspective

Although transplantation experiments conducted in the 1950s established the ability of cells derived from bone marrow and spleen to reverse hematopoietic failure after radiation exposure, HSCs were studied mainly on a morphological basis before the 1960s. A major breakthrough came in 1961, when Till and McCulloch published an article describing the spleen colony-forming assay. This marked the first attempt to describe and quantify stem cell activity *in vivo*. Shortly after this, tissue culture systems were developed that allowed the assay of hematopoietic progenitor cells *in vitro*. These assays provided quantitative results, because of their clonal nature, whereby single cells proliferate to form a colony of differentiating progeny. The mouse became the animal of choice for the early studies of HSCs, because of the ability to obtain bone marrow easily and inexpensively, and to perform transplant studies where the behavior of primitive cells could be studied *in vivo*. In addition, the development of inbred strains of mice had already allowed the definition of the role of the major histocompatibility complex in determining graft acceptance or rejection. This animal model, in combination with the ability of clonal assays to determine enrichment factors for the colony-forming cells, provided the basis for approaches to the characterization and isolation of HSC.

Till and McCulloch demonstrated that the subset of cells able to form spleen colonies was heterogeneous. When spleen colonies were harvested from animals and transplanted into a second set of irradiated recipients, only a subset of colonies could give rise to secondary colonies. This group of cells was also capable of reconstituting lethally irradiated animals. For approximately 20 years, the spleen colony-forming unit was equated with HSC activity. However, in the early 1980s it became clear that this assay measured the behavior of a number of cell types that were not by definition HSCs. Conversely, populations of cells that fit the definition of HSCs, but did not make colonies in the colony-forming unit-spleen (CFU-S) assay, were also identified. Prospective isolation of defined cell populations from the mixture of cell types found in normal bone marrow was required to resolve the differences between true HSC and other types of blood progenitors.

Some of the earliest attempts to isolate stem and progenitor cell subsets from bone marrow involved separating cells based on size, density, and cell cycle characteristics. These experiments demonstrated that the cells that

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produced splenic colonies were largely separable from those that made colonies *in vitro*, and that many spleen colony-forming cells were sensitive to killing by cycle-active drugs, whereas HSC were largely spared. It soon became clear that the only definitive means by which HSC could be distinguished from later stages of development was to evaluate the ability of a transplanted population of cells to maintain production of multiple types of blood cells over many months *in vivo*. Even by this assessment, false indications of stem cell function are possible. Due to the extensive proliferative potential of true stem cells, a single cell remaining within the bone marrow after radiation-induced ablation will proliferate and self-renew sufficiently to dominate long-term hematopoiesis. Although transplanted progenitor cells may provide sufficient hematopoietic function to rescue animals from hematopoietic failure after radiation conditioning, it is necessary to utilize markers to distinguish blood cells derived from the donor graft from those of recipient origin in order to definitively demonstrate HSC function. Examples of such markers include chromosomal abnormalities induced by radiation; retroviral insertion sites; and allelic forms of enzymes, intracellular molecules, and cell-surface antigens. Using these tools, researchers have been able to demonstrate that progenitor cells can be radioprotective without providing the long-term chimerism that is characteristic of HSC. Furthermore, researchers using these tools have shown that a single transplanted or endogenous HSC is sufficient to maintain life-long hematopoiesis.

The development of fluorescence-activated cell sorting (FACS) allowed the isolation of distinct cell populations that were too similar in physical characteristics to be separated by size or density. FACS technology utilizes fluorescent tags to detect surface proteins that are differentially expressed at particular stages of development, allowing fine distinctions to be made among physically homogeneous populations of cells. Although a number of surface proteins are expressed by HSC, there is no single marker that absolutely defines HSC in any species. This makes a multiparameter approach, using positive and negative selection for expression of a variety of markers, a necessary component of any enrichment scheme.

Methods for Enrichment of Mouse Hematopoietic Stem Cells

A method using a combination of negative selection for proteins not expressed by HSC with positive selection for proteins that are expressed has proven to be extremely useful for definition and isolation of HSC from both mouse and human tissue. In the first applications of this technology, both positive and negative selections were applied using FACS. In more recent years, both types of selection have been adapted to magnetic technology. This advance in the field makes simple methods of HSC enrichment accessible to investigators who lack access to sophisticated FACS instruments. However, isolation of true HSC from primitive progenitor cells requires FACS technology, due to the application of intracellular fluorescent probes that indicate cellular quiescence or efflux pump activity. In addition, although magnetic isolation usually maximizes yields at the expense of purity, FACS isolations provide high purity with acceptable yields. A combination of magnetic and FACS selection allows isolation of HSC in a routine manner.

Magnetic Selection Techniques

Regardless of the specific technology used for HSC enrichment, the most critical parameter to be considered is the specific markers to be used in negative and positive selections. For negative selections, a conventional approach has evolved that uses panels of antibodies specific for proteins expressed by most bone marrow cells. Caution must be exercised to select antibodies that do not react with HSC, because this would result in the loss of the HSC during negative selection. It is particularly important to note that developmental stages and activation-specific responses can change the antigenic profile of HSC. For example, the CD34 antigen fluctuates on and off in HSCs during development, as well as during a period of time following the administration of chemotherapy. Other antigens are also known to fluctuate in a similar manner. Therefore, negative selection protocols must be tailored to match the known phenotype of HSCs in the particular tissue of interest. If the investigator wishes to work with tissues for which the antigenic phenotype of HSC has not been described, appropriate assays should be used to evaluate the HSC content of populations fractionated into positive and negative cells, in order to identify antibodies that will be useful in depleting significant numbers of cells without affecting the recovery of HSCs. As discussed previously, the most appropriate assay for this analysis would be a long-term engraftment study, rather than one of the more rapid techniques such as spleen colony formation or *in vitro* culture systems.

Magnetic selections can be accomplished by two general approaches. The first approach, which is best suited for negative selections, involves mixing a cell population marked by the antibodies of interest with a suspension of paramagnetic particles that bind to the antibody tag. Application of a magnetic field will then separate bead-cell aggregates from the unmarked cells, which can be collected by simple aspiration. This technique is rapid, and has the advantage of not perturbing the cells of interest through association with antibody molecules or magnetic particles. Because of this, the magnetic particles of choice will be relatively large (1–5 μm) to facilitate the rapid migration of the labeled cells in the magnetic field.

The second approach to magnetic selection is positive selection of the cells of choice. In this case, particles of a much smaller size (50 nm) are utilized, to minimize potential effects of the antibody–particle complexes on the biology of the selected cells. The small size of the magnetic particles used in positive selection decreases the effectiveness of the magnetic field in attracting the labeled cells. To overcome this limitation, commercial positive selection systems use a flow column packed with a fibrous metal, into which the magnetic field is introduced by induction. This creates a high-flux magnetic field, with very short distances between the labeled cells and the magnetized column matrix, and results in the retention of labeled cells in the column. Once the unlabeled cells are passed through the column and washed out, the column is removed from the magnetic field and the selected cells can be collected.

The combination of negative with positive magnetic selection is a powerful technique for rapid enrichment of a mixture of hematopoietic stem and progenitor cells. These cells may be used directly in studies evaluating hematopoietic function *in vivo* or *in vitro*, or may be further processed to achieve higher purity of functionally-distinct subsets of HSCs or progenitor cells (see later discussion). A specific method, originally reported using negative and positive selection by FACS, can easily be modified to substitute magnetic selections using the same combinations of antibodies as originally described (Table 50-1). Unconjugated rat antibodies are used in combination with magnetic beads conjugated to anti-rat immunoglobulin for negative selection, while avidin-biotin selection systems work very well for positive selection using biotinylated antibodies followed by avidin-conjugated microbeads. HSCs and progenitor cells can be enriched routinely by a factor of 1000 using this technique.

TABLE 50-1 Antibody Specificities for use in a Negative–Positive Magnetic Enrichment Protocol

Negative Selection		
Antigen	**Antibody**	**Lineage**
CD11b	M1/70	myeloid[a]
Ly6-G	RB6-8C5	myeloid
CD45 (B220 isoform)	RA3-6B2	lymphoid
CD2	RM2.2	lymphoid
CD3	KT3	lymphoid
CD5	53–7.3	lymphoid
CD8	53–6.7	lymphoid
TER-119	TER-119	erythroid
Positive Selection		
Antigen	**Antibody**	**Lineage**
Ly6A/E	E13 161-7	various[b]

a. CD11b is expressed by mouse fetal liver stem cells, and should not be included in negative selections from this tissue.
b. Ly6A/E (Sca-1) is expressed by lymphoid lineage cells, as well as by hematopoietic stem and progenitor cells. Depletion of the lymphoid lineage cells during negative selection allows positive selection based on Ly6A/E to yield a highly-enriched population of stem and progenitor cells.

Fluorescence-activated Cell Sorting Selection

Although magnetic enrichment strategies are sufficient when mixed populations of cells are an adequate product, the more refined approaches to HSC and progenitor cell characterization require FACS selection. Modern commercial FACS instruments can separate populations of cells based on 15 parameters, allowing the simultaneous application of positive and negative selection for a variety of surface markers. High-speed sorting improves the yield of cells obtained from this approach, and purity is very high. Workers in the field often utilize a negative selection prior to FACS sorting, because a good negative selection reduces the cellularity of the sample, and thus the time needed for sorting, by a factor of 50 to 100 without adversely affecting the yield of HSC. Samples subjected to positive magnetic selection can subsequently be processed to isolate specific cellular subsets by FACS. Pre-enrichment of target populations prior to FACS sorting can have a significant impact on the final purity of the isolated cell populations.

Selection Based on Cell-Surface Antigen Expression. Recent studies have described antigenic profiles of a variety of progenitor cell subsets in the mouse. Progenitors restricted to lymphoid, myeloid, and erythroid lineages have been characterized for prospective isolation using FACS technology. In addition, the hierarchy of HSCs, which includes cell populations possessing more or less potential for self-renewal, has been fractionated based on surface antigen expression.

Selection Based on Supravital Stains. The combination of multiple cell surface markers with supravital fluorescent dyes that indicate cell function has become a method of choice for isolating HSCs. Baines and Visser first utilized the fluorescent DNA probe Hoescht-33342 to separate quiescent cells from the bone marrow. More recently, red and blue emissions from Hoescht-33342 have been used to define a small subset of bone marrow cells, called the side population, which comprise approximately 0.07% of the whole bone marrow, and are highly enriched for long-term reconstituting HSC. Recently, the molecular basis for the ability of Hoescht-33342 to identify HSC has been suggested to be due to its role as a substrate for the multidrug resistance pump ABCG2. Interestingly, expression

of ABCG2 is a characteristic also observed in embryonic stem cells, suggesting that expression of this protein may represent a general marker of stem cells.

A second supravital fluorescent probe, rhodamine-123 (Rh-123), has also proven useful for distinguishing subsets of primitive stem cells. Although Rh-123 is most widely used as a substrate to evaluate multi-drug resistance pump activity, a second characteristic of the probe is its affinity for the inner mitochondrial membrane. Hence, the intensity of Rh-123 staining reflects a balance between efflux by specific molecular pumps, and accumulation in mitochondrial membranes. Because Rh-123 is a charge-sensitive probe for mitochondria, accumulation of the probe is directly proportional to cellular activation state, and inversely proportional to cellular efflux activity. In combination, these two selective criteria achieve a remarkable segregation of stem and progenitor cells previously isolated by negative/positive selection based on antigen expression. Primitive HSCs, which express high levels of efflux pump activity, and contain few activated mitochondria, accumulate very little Rh-123 during a 30-minute labeling period. In contrast, progenitor cells which have become activated by the differentiation process contain many activated mitochondria, and have down-regulated efflux activity.

The combination of cell surface and metabolic markers provides a means to enrich HSCs to the point that fewer than 10 cells are required to reconstitute hematopoiesis in irradiated mice. In addition, enrichments based on antigenic expression combined with Rh-123 allow simultaneous isolation of Rh-123^{low} HSC, as well as Rh-123^{high} primitive progenitor cells, allowing for direct comparisons of the functions and molecular signatures of these two closely-related populations of cells.

ISOLATION OF HEMATOPOIETIC STEM CELLS FROM HUMANS

Historical Perspective

In contrast to the mouse, in which HSC behavior can be defined in transplant experiments, defining and quantifying human HSCs has been difficult because transplantation experiments using limiting dilution or competitive repopulation approaches are impossible to perform in humans. *In vitro* colony-forming assays were once routinely used to estimate the number of HSC in a bone marrow or peripheral blood stem cell harvest. However, with the recognition that these assays fail to distinguish different classes of primitive stem cells that have distinct functions on transplantation, other assays were developed. Several *in vitro* assays, including the long-term culture initiating cell assay, and the cobblestone area forming cell assay, have been used to measure human stem cell number and behavior.

Researchers have addressed the inability to study HSC engraftment behavior in humans by developing xenogeneic transplant models. Several models which utilize immunodeficient mice as transplant recipients have been developed to measure engraftment and self-renewal of human HSC *in vivo*. The non-obese diabetic-severe combined immunodeficiency (NOD-SCID) and the compound mutant X-linked SCID-recombinase-activating gene mouse models have emerged as standard assays that allow engraftment of human cells in mouse bone marrow. Ultimately, however, studies that depend on xenogeneic transplantation rely on inference, and stem cells may behave differently in systems that are more genetically compatible.

The first attempts to separate human HSCs from bone marrow mirrored earlier studies in mouse. The recognition that primate hematopoietic progenitor cells expressed CD34 provided a way for scientists to study the HSC compartment in humans. Using techniques pioneered in the mouse, researchers used negative selection against lineage antigens, and positive selection for CD34. The CD34+ subset was found to be heterogeneous, with most of the cells being progenitors for the erythroid, lymphoid, or myeloid lineages. Further enrichment of HSCs was obtained by positive selection for additional surface antigens, in combination with metabolic markers.

CD34 as the Primary Marker of Human Hematopoietic Stem Cells

The utility of CD34 as a general marker of HSCs in human tissue was initially controversial, due to research groups that published conflicting results regarding the isolation of mouse HSC based on CD34 expression. This issue was resolved in a series of experiments by Ogawa and colleagues, in which the expression of mouse CD34 was shown to be modulated from negative to positive, depending on the proliferative state of the HSC population under analysis. Thus, although most HSCs found under normal steady state conditions in adult mouse bone marrow lack CD34 expression, stimulation *in vivo* by chemotherapy, or *in vitro* by cytokine stimulation, resulted in recovery of most HSC activity among CD34+ cells. The expression of CD34 was again lost, as the cells engrafted in marrow and returned to steady state conditions. Similar observations were reported in an analysis of mouse ontogeny. Further experiments showed that CD38, which is absent from human HSC but present on mouse HSC under steady state conditions, also modulates during recovery from chemotherapy, or during mobilization induced by granulocyte colony-stimulating factor (G-CSF). Thus, in the mouse, the expression of CD34 and CD38 reflect the activation state of the HSC. Interestingly, an analysis of the expression of human CD34 in a transgenic mouse model demonstrated that the human and mouse genes are

differentially regulated, suggesting that the current practice of human bone marrow transplantation using only CD34+ cells is justified, in spite of the mouse data. Since the initial data from the mouse model could be interpreted to indicate that human patients receiving CD34+ transplants are missing an important subset of HSC, it is important to learn that CD34 expression is a characteristic of most HSC in humans.

The question of lineage marker fidelity between mouse and human is biologically interesting, because it might imply important functions for these molecules in the behavior of HSC subsets. The functions of these molecules in mice and humans must be identified, so that the gene program that specifies HSCs and their functions can be better understood. Enriched populations of CD34+ cells have been used clinically for transplantation in humans following chemotherapy. Enriching marrow progenitors and purging T-cells has been used to prevent graft versus host disease in allogeneic transplantation, and as a means to enrich human HSC for gene therapy applications.

HEMATOPOIETIC STEM CELLS AS A PARADIGM FOR STEM CELL BIOLOGY

Robust production of blood is essential for survival, because of the critical roles blood plays in oxygenation of tissues, control of vascular integrity, and distribution of cells responsible for immunity to infection. Furthermore, the large total number of blood cells, coupled with relatively short cellular lifespans, results in a need for ongoing replacement of senescent cells. The sustained daily rate of blood cell production reflects the potential of the stem cells that drive the process of hematopoiesis. These extreme demands of proliferation and differentiation, coupled with a long history of successful transplantation in humans, make the HSC a gold standard for all forms of stem cell therapy. The development of methods to enrich this class of stem cells serves as a robust paradigm by which other applications of stem cell therapy might be measured. It is entirely possible that organ systems, such as the central nervous system, while harboring cells with stem cell-like characteristics, have not been subjected to evolutionary pressure favoring rapid replacement of dying cells. It will, therefore, be critical to understand more of the regulatory control over self-renewal, proliferation, and differentiation of HSC in order to facilitate the application of stem cell therapies for tissues lacking the demands of blood production.

ACKNOWLEDGMENT

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KEY WORDS

Antigen A substance, usually a protein, that serves as a target for the binding of an antibody molecule. Antigens can exist as free proteins, or can be found associated with the outer surface of cells. Stem cells express a pattern of antigens that can be detected using fluorescent-labeled antibodies using a FACS instrument, or by unlabeled antibodies using magnetic selection methods.

Fluorescence-activated cell sorting (FACS) A technology that evolved from a combination of instruments designed to count blood cells and ink-jet printers. FACS combines detection of fluorescent indicator molecules with the physical isolation of single cells that are labeled with the fluorescent molecules. This technology allows separation of stem cells from a mixed population of other cell types, provided that indicator molecules, such as fluorescent-labeled antibodies that distinguish the stem cells, are available.

Hematopoietic progenitor cell A type of cell found in bone marrow and other tissues that have lost the potential for self-renewal, but is still multipotent (can produce some or all types of blood cells).

Hematopoietic stem cell A type of cell found in bone marrow and other tissues that possesses the defining characteristics of self-renewal (divides to produce more stem cells) and multipotency (produces all types of blood cells).

Spleen colony-forming assay The first biological assay used to detect hematopoietic stem cells. The assay consists of counting the number of macroscopic splenic nodules that form following proliferation of stem and progenitor cells that lodge in the spleen after intravenous injection into an irradiated animal. The cells that initiate these nodules are termed colony-forming units-spleen (CFU-S), because it was initially not certain that each nodule represented the outgrowth of a single cell versus that of a cluster (unit) of cells.

Supravital fluorescent probe A fluorescent molecule that can enter a living cell and bind to specific subcellular components, such as DNA, RNA, or organelle membranes. These probes can be used in combination with labeled antibodies and FACS in order to identify and isolate stem cells.

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Chapter 51

Microarray Analysis of Stem Cells and Differentiation

Howard Y. Chang and Xin Chen

INTRODUCTION

For the past several decades, biologists have only been able to tackle the analysis of one or a few genes at a time. However, the advent of complete genomic sequences of over 800 organisms (including the human, mouse, and zebrafish genomes), and the development of microarray technology have revolutionized molecular biology. Microarrays enable biologists to perform global analysis on the expression of tens of thousand of genes simultaneously, and they have been widely used in gene discovery, biomarker determination, disease classification, and studies of gene regulation. Expression profiling using microarrays is generally considered "discovery research," although it can also be a powerful approach to test defined hypotheses. One advantage of microarray experiments is that, at the outset, microarray experiments need not be hypothesis driven. Instead, it allows biologists a means to gather gene expression data on an unbiased basis, and can help to identify genes that may be further tested as the targets in hypothesis-driven studies.

Overview of Microarray Technology

There are two major microarray platforms that have been widely used: cDNA microarrays; and oligonucleotide microarrays.

cDNA Microarrays

The principle of cDNA microarray is illustrated in Figure 51-1. In brief, cDNA clones, which generally range from several hundred base pairs to several kilobases, are printed on a glass surface, either by mechanical or ink-jet microspotting. Sample RNA and a reference RNA are differentially-labeled with fluorescent Cy5 or Cy3 dyes, respectively, using reverse transcriptase. The subsequent cDNA are hybridized to the arrays overnight. The slides are washed and scanned with a fluorescence laser scanner. The relative abundance of the transcripts in the samples can be determined by the red/green ratio on each spotted array element.

One of the limitations of cDNA microarray has been that it required relatively large amount of total RNA ($\geqslant 10\mu g$) for hybridization. However, significant progress has been made recently for linear amplification of RNA, generally based on Eberwine's protocol. In this case, RNA is converted into cDNA with oligo dT primers which contain T7 RNA polymerase promoter sequence at its 5′ end. The cDNA can be subsequently used as the template for T7 RNA polymerase to transcribe into anti-sense RNA. The linear amplification protocol can produce 10^6-fold amplification. Therefore, only very small amounts of samples are required in modern microarray experiments.

There are several advantages to cDNA microarrays. The two-color competitive hybridization can reliably measure the difference between two samples, since variations in spot size or amount of cDNA probe on the array will not affect the signal ratio. cDNA microarrays are relatively easy to produce. In fact, the arrayer can be easily built and allow the microarrays to be manufactured in university research laboratories. Also, cDNA microarrays are, in general, much cheaper compared with oligonucleotide arrays, and are quite affordable to most research biologists.

There are also some disadvantages for this system. One is that the production of cDNA microarray requires the collection of a large set of sequenced clones. The clones, however, may be misidentified or contaminated. Second, genes with high sequence similarity may hybridize to the same clone, and generate cross-hybridization. To avoid this problem, clones with 3′ end untranslated regions which, in general, are much more divergent compared with the coding sequences, should be used in producing the microarrays.

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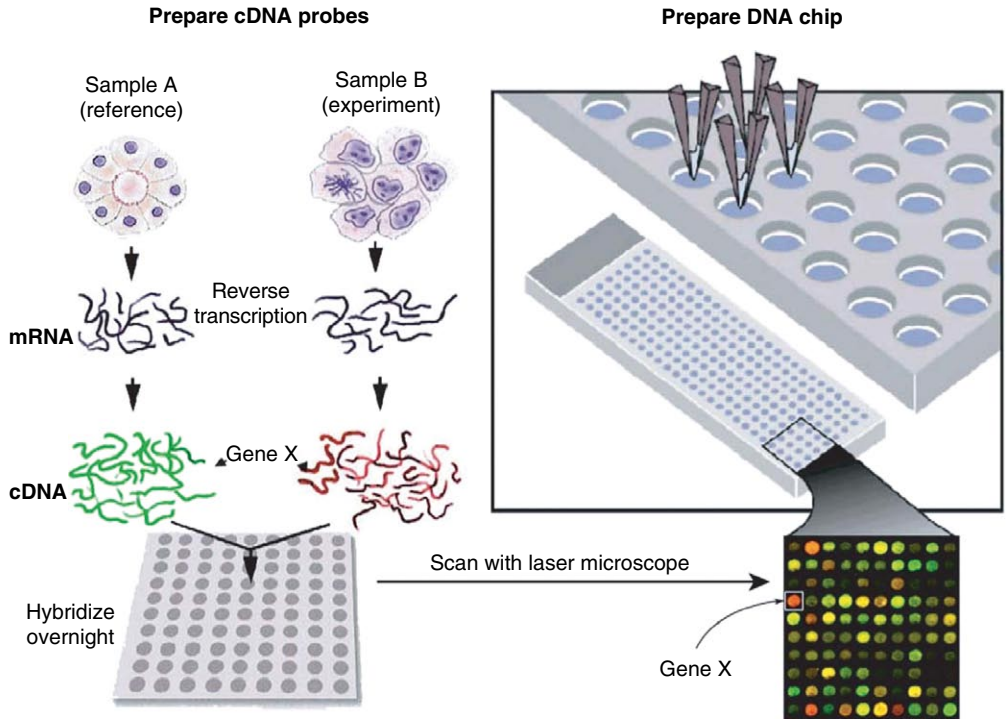


FIGURE 51-1 Principle of cDNA microarrays. PCR products are printed onto glass slides to produce high density cDNA microarrays. RNA is extracted from experimental samples and reference samples, and differentially labeled with Cy5 and Cy3, respectively, by reverse transcriptase. The subsequent cDNA probes are mixed and hybridized to cDNA microarray overnight. The slides are washed and scanned with fluorescence laser scanner. The relative red/green ratio of gene X indicates the relative abundance of gene X in experimental samples versus reference.

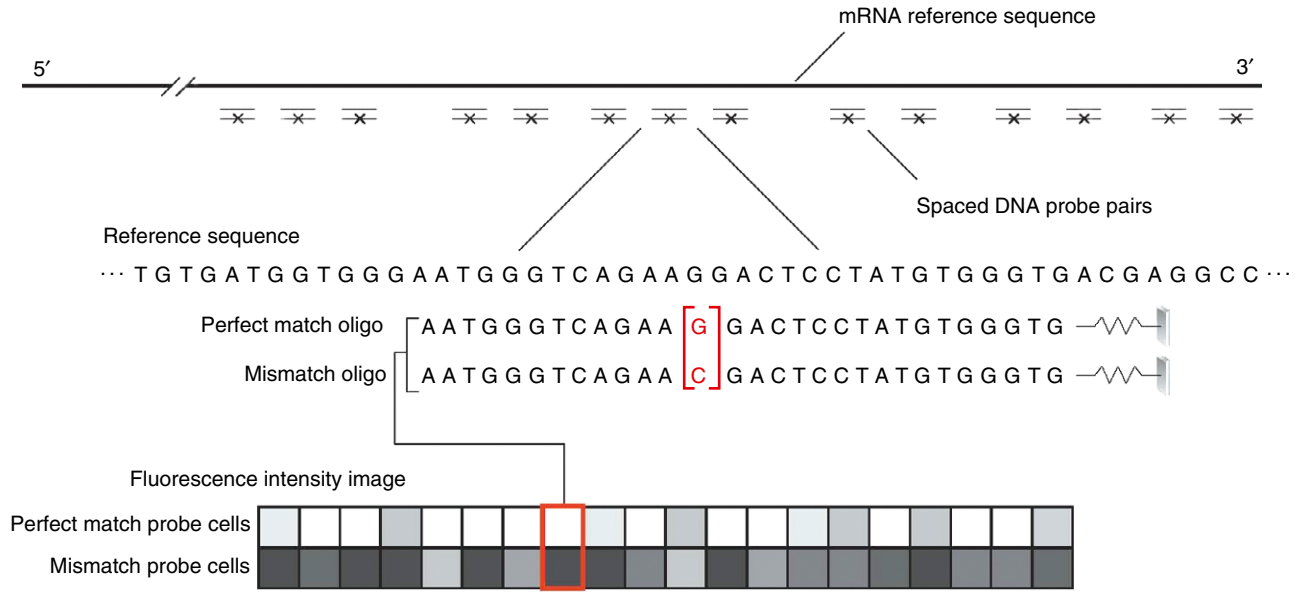


FIGURE 51-2 Principle of Affymetrix GeneChip. For each gene, approximately 20 distinct and minimally overlapped 25-mer oligonucleotides are selected and synthesized on the array. For each oligonucleotide, there is also a paired mismatch control oligonucleotide probe, which differs from the perfect match probe by one nucleotide in the central position. Comparison of the hybridization signals from perfect match oligonucleotide with the paired mismatch oligonucleotide allows automatic subtraction of background. Figure kindly provided by Affymetrix, Santa Clara, California.

Oligonucleotide Arrays

The most widely used oligonucleotide arrays are GeneChips produced by Affymetrix, which uses photolithography-directed synthesis of oligonucleotides on glass slides. The Affymetrix GeneChip measures the absolute levels for each transcript in the sample. The principle of the +Affymetrix GeneChip is shown in Figure 51-2. In general, for each transcript, approximately 20 distinct and minimally overlapped 25-mer oligonucleotides are selected and synthesized on the

array. For each oligonucleotide, there is also a paired mismatch control oligonucleotide, which differs from the perfect match probe by one nucleotide in the central position. Comparison of the hybridization signals from the perfect match oligonucleotide with the paired mismatch oligonucleotide will allow automatic subtraction of background.

There are several advantages of oligonucleotide arrays. The sequences of the oligonucleotide arrays are determined by sequencing information from the database, and are synthesized *de novo* for the arrays. Therefore, there is no need to validate cDNA clones or PCR products that are used to print the cDNA microarray. The use of multiple oligonucleotides for each transcript also allows for the detection of splice variants, and helps to distinguish genes with high sequence similarities.

The major disadvantage of oligonucleotide arrays is that they can only be produced by commercial manufacturers, and these prefabricated GeneChips are still very expensive. Currently, there are few studies that have systematically compared the two microarray platforms. Both cDNA microarrays and oligonucleotide arrays have been widely-used in biological research and produce data.

Next, we discuss some basic issues of microarray experiments, including experimental design, data analysis, and gene validation. Although we focus on cDNA microarrays, most of the general principles can be applied to both cDNA microarrays and oligonucleotide arrays.

Experimental Design

cDNA microarrays use two-color competitive hybridization; therefore, the design of experiments (i.e., which samples are labeled for each color) is very important in the subsequent data interpretation. There are two commonly-used experimental designs; in this chapter, we refer to them as "type I" or "type II" designs.

In the type I experimental design, the sample RNA (e.g., drug-treated cells, diseased tissues) is labeled with one dye, and another sample RNA (for example, mock-treated cells, normal tissues) is labeled with another dye. The two sample cDNA probes are mixed and cohybridize to microarray. The data analysis for the type I design can be quite straightforward. The relative red/green ratio represents the relative up-regulation or down-regulation of each gene. This design is most suited for experiments with a few perturbations; for example, to identify genes response to certain stimulus, or genes affected by a genetic mutation. However, it is difficult to apply this pair-wise type I design to a complicated system. For example, to identify genes differentially-expressed in diseased and normal tissues, it may not be appropriate to compare diseased tissues versus corresponding normal tissues from the sample patients. Gene expression patterns in normal and diseased tissues are affected by many factors, including patient variation (ethnicity, sex, age, genetic background), sample variation (proximity to disease, anatomic location, and developmental range), as well as heterogeneity of cell populations within the samples. In the simple pair-wise type I design, it is impossible to distinguish all these variations within both normal and diseased tissues. Another drawback of the type I experiment is that it cannot accurately measure the relative abundance of a transcript which is not expressed, or is expressed at very low levels in Cy3 labeling, as this would produce a green channel signal very close to the background signal.

Type II experimental design avoids most of the problems with the simple pair-wise type I design. In the type II design, a common reference is used in Cy3 channel labeling, and each sample is compared with the same common reference. The two most important criteria for selection of common reference are gene coverage ("light up" most of the spots on the array), and reproducibility (can be relatively easily reproduced with minimum batch to batch variation). In most cases, the reference pool RNA is derived from a mixture of tumor cell lines, and they are commercially available from Stragene and Clontech. Cell lines from different histological origins ensure the complexity of the reference. This provides a relatively good coverage of the spots on the array. Cell lines can be recultured to produce more reference RNA; however, growth conditions need to be tightly-controlled to reduce batch-to-batch variation. The greatest advantage of the type II experimental design is that it allows the cross-comparison of many samples collected over long period of time, by different persons, as well as samples from different sources.

Another situation in which type II designs are widely used is in time series experiments, i.e., to study gene expression variations in response to a stimulus, for example, drug treatment, or growth factor stimulation. A simple pair-wise type I design using treated cells against untreated cells may be quite straightforward. However, some of the genes that are induced by the stimulus may be not expressed in the untreated cells. It is difficult to obtain an accurate description of the expression profile changes over time for these genes. A better design is a type II design, in which the cells from different time points are mixed and used as reference. All the samples, including untreated cells ($t = 0$), can then be compared with this reference. This average $t = 0$ measurement can then be subtracted from each subsequent time point measurement, in order to depict the temporal response patterns of expression relative to $t = 0$ as the baseline. This design ensures that all the transcripts present at different time points are represented in the reference RNA, and therefore the design accurately describes the temporal changes in response to the stimulus.

Although there are many advantages with the type II design, one has to realize that the design may make the experiments more complicated, and sometimes inefficient. In all the analyses, the baseline reference signals have to be subtracted in order to extract biologically meaningful data. This undoubtedly will add more data variations. So the

proper selection of experimental design is one of the most important steps toward the success of experiments.

Data Analysis

Microarray experiments produce large amounts of data, for example, 30 000 genes × 100 samples will generate three million data points. Data analysis may be one of the most challenging issues facing biologists in microarray experiments. Microarray data are often noisy and not normally distributed, and usually have missing values in the matrix. During the past several years, with the combined efforts of biologists, statisticians, and computer scientists, there has been great progress in the bioinformatic techniques that can be used for the analysis of genome-wide expression data. However, there is no standard or one-size-fits-all solution for interpretation of microarray data.

Current methods for microarray data analysis can be divided into two major categories: supervised and unsupervised methods. Supervised approaches try to identify gene expression patterns that fit a predetermined pattern. Unsupervised approaches characterize expression components without prior input or knowledge of predetermined pattern.

Supervised Analysis

The purpose of supervised analysis is to identify genes that are differentially-expressed between groups of samples, as well as to find genes that can be used to accurately predict the characteristics of groups. There are many statistical tests developed for identification of differentially-expressed genes in microarray data, e.g., the *t*-test for detecting significant changes between repeated measurements of a variable in two groups; and an ANOVA (Anaysis of Variance) F statistic for detecting significant changes in multiple groups. These tests involve two parts: calculating a test statistic, and determining the false discovery rate (FDR), or the significance of the test statistic. Here are some commonly used statistical methods for two group comparison.

Non-parametric T-test

In a non-parametric *t*-test, the statistical significance of each gene is calculated by computing a *p* value for it, without assuming a specific parametric form for the distribution of the test statistics. To determine the *p* value, a permutation procedure is used in which the class labels of the samples are computed (10 000 to 500 000 times), and for each permutation, *t* statistics are computed for each gene. The permutation *p* value for a particular gene is the proportion of the permutations in which the permutated test statistic exceeds the observed test statistic. A *p* value cut-off can be chosen for the dataset. And the FDR = number of genes test × cut-off *p* value/gene can be declared to be significant.

Wilcoxon (or Mann-Whitney) Rank Sum Test

This test rank transforms the data and looks for genes with a skewed distribution of ranks. The rank sum transform smooths the data, by reducing the effect of outliers. This method is proven to be superior for decidedly non-normal data. In general, the Wilcoxon rank sum test has been shown to be most conservative, with a lower FDR.

Ideal Discriminator Method

This method is based on the similarity of gene expression patterns on the array to a theoretical pattern that clearly discriminates between two groups. It potentially allows more flexibility in defining more complex theoretical pattern behavior.

Significant Analysis of Microarray (SAM)

SAM uses a statistic that is similar to a *t*-statistic. However, it introduces a fudge factor at the denominator when calculating the *t* statistic, therefore, it underweights those genes which have relatively small magnitudes of difference and small variations within groups. It also permutes the whole dataset and sets a threshold for an FDR, instead of assigning an individual *p* value to each gene.

Overall, each statistical method has its own advantages. Each biologist may need to choose proper tests for his/her own dataset, and in some cases, possibly try different statistical tests. In a situation where the most reliable list of genes is desirable, the best approach may be to examine the intersection of genes identified by different statistical tests, or by the more conservative rank sum test and non-parametric *t*-test. However, if a more inclusive list of genes is desired, a higher *p* value cut-off or SAM test may be more appropriate.

Unsupervised Analysis

Users of unsupervised analysis try to find internal structure in the data set without any prior input knowledge. Here are some widely used analytical methods.

Hierarchical Clustering

Hierarchical clustering is a simple but proven method for analyzing gene expression data by building clusters of genes with similar patterns of expression. This is done by iteratively grouping together genes that are highly-correlated in their expression matrix. As the result, a

dendrogram is generated. Branches in the dendrogram represent the similarities among the genes; the shorter the branch, the greater similarity of the gene expression pattern. Hierarchical clustering is also popular because it helps to visualize the overall similarities of expression profiles. The number and size of expression patterns within a dataset can be quickly estimated by biologists.

Self-organizing Maps (SOM)

In SOM, each biological sample is considered as a separate partition of the space, and after partitions are defined, genes are plotted using the expression matrix as a coordinate. To initiate SOM, the number of partitions to use must first be defined by the users as an input parameter. A map is set with the centers of each partition (known as centroids) to be arranged in an initially arbitrary way. As the algorithm iterates, the centroids move toward randomly chosen genes at a decreasing rate. The method continues until there is no further significant movement of these centroids. The advantage of SOM is that it can be used to partition the data with easy two-dimensional visualization of expression patterns. It also reduces computational requirements compared to other methods. The drawbacks of the method are that the number of partitions has to be user-defined, and genes can only belong to a single partition at a time.

Singular Value Deposition (SVD)

SVD is also known as "principle-components analysis" by statisticians. SVD transforms the genome-wide expression data into diagonalized "eigengenes" and "eigenarrays" space, where the eigengenes (or eigenarrays) are the unique orthogonal superpositions of the genes (or arrays). Sorting the gene expression data according to the eigengenes and eigenarrays will reduce the features of the data to their principal components, and may help to identify the main patterns within the data. Importantly, SVD is also a powerful technique to capture any patterns within the data that may be due to experimental artifacts. For example, array hybridization on different days, or hybridization on different batches of arrays, occasionally gives slight differences in gene expression patterns. SVD can also remove the artifacts without removing any genes or arrays from the dataset.

Post Data Analysis

Gene Ontology

Microarray experiments generally generate large amounts of data. Even after statistical analysis, one may identify gene clusters that consist of hundreds of genes which are differentially expressed. One of the most commonly used methods to annotate the gene function is through Gene Ontology (GO, http://www.geneontology.org/). GO classifies gene function according to three organizing principles: molecular function; biological process; and cellular component. There are thousand of GO terms under each principle. Currently there are over 9000 GO terms listed. Clusters of genes can therefore be classified by GO terms. When certain GO terms are statistically-enriched in a cluster, it may suggest possible functional significance of the cluster of the genes. However, one has to realize the limitations of GO annotation, as our understanding of the functions of the genes are still incomplete, and many genes may have multiple functions.

Gene Module Analysis

Just as GO term analysis builds on pre-existing knowledge to refine the interpretation of microarray data, one can interrogate the global gene expression profile with respect to known sets of genes by gene module analysis. Specifically, this class of methods asks whether the genes whose level of expression changes in an experiment are similar to those which have been observed in another setting. The gene modules may be defined by function (e.g., GO terms, or other annotations), the presence of specific cis-regulatory motifs for transcription factor or miRNA binding, or known responsiveness to specific signaling pathways or drugs. The results of such comparisons can then generate many testable hypotheses. Gene module analysis can be accomplished by several algorithms, including the Gene Module Map, Gene Set Enrichment Analysis (GSEA), and others. While many of these algorithms have been successfully used, care and the use of proper controls should always be applied in interpreting their results.

Regulatory Networks

Regulatory networks aim to move microarray analysis from correlation to causality. For instance, genes A and B may be co-expressed because A activates B. This inference of causality may be justified by additional computational or experimental evidence, such as the fact that gene B contains binding sites for the protein encoded by gene A, that protein A occupies the promoter of gene B, that activation of gene A precedes that of gene B, or that expression of gene B is decreased when gene A is inactivated. Regulatory network analysis refers to a class of algorithms that can integrate multiple types of data—for instance, gene expression, ChIP-chip, temporal series, perturbational data—and predict regulatory relationships. Probabilistic models, based on iterative training and cross-validation, are often used to build regulatory networks. The performance of regulatory networks is typically scored by their false discovery rates, and by their ability to predict novel regulatory relationships that are subsequently verified by experimentation.

Confirmation Studies

It is very important to independently verify array observations. There are two approaches: in silico analysis, and experimental validation.

The *in silico* method compares array results with information available in the literature or other independent array expression databases. Agreement between array results from other groups, especially using different array platforms, will validate the general performance of the system and provide confidence in the overall data.

Experimental validation uses an independent experimental method to assay the expression levels of genes of interest, preferably on another sample set than the one used in the microarray analysis. The methods that have been used widely depend on the specific scientific questions. Commonly used techniques include at the mRNA level: semi-quantitative RT-PCR; real-time RT-PCR (Taqman, Applied Biosystems); Northern blot; *in situ* hybridization (ISH). At the protein level: Western blot; fluorescence activated cell sorting (FACS); enzyme-linked immunoabsorbent assays; immunofluorescence; and immunohistochemistry (IHC). Both ISH and IHC can be performed in a high-throughput manner via tissue arrays. In addition, both methods provide additional information on the anatomical relationship and cellular origin of gene expression programs.

Other Applications of Microarray Technology

In addition to studying global gene expression programs, microarray technology has been applied in many other areas, including chromatin IP, microRNA expression, and comparative genomic hybridization.

Array-based Comparative Genomic Hybridization (aCGH)

Microarrays can be used to detect DNA copy number, and other genomic aberrations, in a high-throughput fashion, which is generally referred as array CGH or aCGH. The high density of probes on microarrays can enable detection of small deletions and amplifications. Genomic DNA of stem cells can be compared to reference DNA from somatic cells (e.g., normal blood) by two-color labeling and competitive hybridization in an analogous fashion to mRNA expression arrays. This application is particularly valuable for verifying the genomic integrity of stem cells that may be used for therapeutic purposes.

Chromatin Immunoprecipitation Followed by Microarray Analysis (ChIP-chIP)

ChIP-chIP employs antibodies specific to a candidate regulator for genome-scale chromatin-immunoprecipitation, combined with microarrays spotted with intergenic sequences to identify their bound targets. Protein-bound DNA fragments retrieved by the antibody, typically ~500 to 1000 base pairs, are hybridized to microarrays to identify the retrieved sequences. ChIP-chIP analysis has been useful in stem cell biology for identifying direct targets of transcription factors and chromatin remodeling complexes that are important for pluripotency and differentiation, such as those of the Polycomb Group Proteins, Oct-4, Nanog, and Myc, among others.

MicroRNA Arrays

MicroRNAs (miRNAs) are single-stranded RNAs, typically with ~21 nucleotides, non-coding RNAs. Studies have demonstrated that microRNAs play important roles in multiple cellular processes, including stem cell self-renewal and differentiation. MicroRNA arrays have been applied to study the variation of miRNA expression in cells. The principal MicroRNA array study design and procedures are similar to RNA microarrays, except the probes on the arrays are generally much shorter.

Examples of Microarray Experiments

Classically, differentiation is defined by the expression of lineage-specific markers, the appearance of unique cell morphologies, or the acquisition of specific biologic functions (e.g., hemoglobin synthesis in red blood cells). From a genomic perspective, differentiation can be considered as sets of gene expression programs; these gene expression programs may be self-reinforcing, sequential, or mutually exclusive, depending on the specific biological context. Thus, the biological state of stem cells, and their subsequent differentiated states, is highly amenable to microarray analysis. In exploring stem cell biology, expression profiling offers a decided advantage as an experimental approach, because no specific assumptions are necessary at the outset. By observing the activity of the entire genome, one can ask what major features define stem cells and characterize their differentiation programs toward specific lineages. Here we highlight several examples in the literature that apply microarray technology to tackle several of the main questions in stem cell biology.

Identification of "Stemness"

Stem cells share certain biological properties—the capacity for self-renewal and multipotency; what are the molecular programs that underlie these properties? Several investigators have approached this problem by comparing the gene expression profiles of several embryonic and adult stem cells. By comparing the intersection of the relatively enriched genes in stem cells, a set of genes that are shared by several stem cells has been identified. It is reassuring that

traditional markers of stem cells, e.g., CD34 for marrow-derived hematopoietic stem cells, were also found to be enriched in the stem cell transcriptome by microarray analysis. In addition, these results provide several candidate pathways that may be involved in regulating maintenance of stem cell fate. However, comparison of the genes identified in the two initial studies demonstrated little overlap in genes, and additional studies following the same strategy of intersecting stem cell enriched genes from multiple tissue types failed to identify a core set of conserved genes. These conflicting findings raised the importance of cross-validation in microarray studies; that is demonstrating the validity of a finding in multiple samples or data sets.

The complexity and subtlety of putative "stemness" programs that may be evident in several types of stem cells may be better approached using the gene module approach than analyses focused on individual genes. Recently, Wong *et al.* employed the module map approach to systematically compare gene expression programs in hundreds of profiles of embryonic stem cells, adult tissue stem cells, and also cancer stem cells. In contrast to inconsistencies with gene-by-gene approaches, the module map identified sets of genes that were supported by experiments from independent laboratories, and using completely different experimental approaches. For instance, a set of genes was found to distinguish embryonic stem cells from other cells by differential mRNA expression in data generated by several laboratories, and the same set of genes were enriched for those that are directly occupied by stem cell transcription factors Oct-4 and were dependent on Oct-4 for their expression. This study further showed that two different transcriptional programs fundamentally distinguished embryonic stem cells and adult tissue stem cells, and that cancer stem cells resembled embryonic stem cells rather than adult tissue stem cells. Guided by this information, Wong *et al.* were able to reprogram differentiated somatic human cells directly into human cancer stem cells by introduction of three genes, which reactivated the embryonic stem cell-like transcriptional program. This example, linking embryonic to cancer stem cells, demonstrates the possible advantages of integrating multiple heterogeneous sources of genome-scale data by modular approaches that consider sets of genes rather than single genes.

Differentiation

The excitement about stem cells arises from their ability to differentiate into many lineages and cell types, but on a practical level, the pluripotency of stem cells present experimental difficulties in guiding and assessing their development into particular lineages *in vitro* and *in vivo*. Traditionally, one may rely on certain well-established markers of the cell types in question, and in some instances, one may verify the ability of one or more stem cells to repopulate a compartment by reconstitution experiments (e.g., reconstitution of peripheral blood cells by transplantation of HSCs). However, these approaches rely on a relatively small number of protein markers, or are laborious and time-consuming. The specificity of lineage markers has been explored to a limited extent in many cases, and many cell types of biological and medical interest do not have well-defined markers. For instance, CD34, the classical marker for HSC, is also present in a number of other cell types and neoplasms, (e.g., endothelial cells, and the fibrohistiocytic tumor dermatofibrosarcoma protuberans). Expression profiling of stem cells and their differentiated derivatives can help to identify the direction and progress of the differentiation program, as well as the interrelationships of the possible differentiation states to one another.

In a prime example of this strategy, Xu *et al.* observed that exposure of human embryonic stem cells to bone morphogenetic protein 4 (BMP4) induced a substantial number of trophoblast markers, including the placental hormone human chorionic gonadotropin. Thus, BMP4 is probably a key molecular switch that guides the first differentiation event of embryonic stem cells toward this extraembryonic lineage, and BMP4-treated ES cells provide a simple system to derive human trophoblastic cells for studying maternal–fetal interactions. In another powerful use of microarray technology, cell surface markers can also be identified in a high throughput fashion, by isolating messenger RNA associated with membrane-bound polysomes; hybridization of such selected RNAs to microarrays allows rapid identification of membrane proteins that are likely to be useful lineage markers and receptors that respond to environmental stimuli.

Stem Cell Niches

In many of the medical applications based on stem cells, introduction of stem cells into the diseased tissue somehow allows the engraftment and differentiation of the stem cells to replace the deficient cell type. Underlying these startling observations is the concept that stem cell behavior is intimately related to, and controlled by, the microenvironment. Specifically, the microenvironment that maintains the biological properties of the stem cell is termed its niche, and depletion of endogenous stem cell populations in disease produces empty specific niches that guide new, exogenous stem cells to differentiate into the missing cell types.

Thus, one of the central questions in stem cell biology is the molecular features of the niches that govern the behavior of fetal and adult tissue-specific stem cells. Although some of these key molecules have been identified genetically in amenable organisms, many of the molecular details that define stem cell niches, especially in mammalian systems, are incompletely understood, and

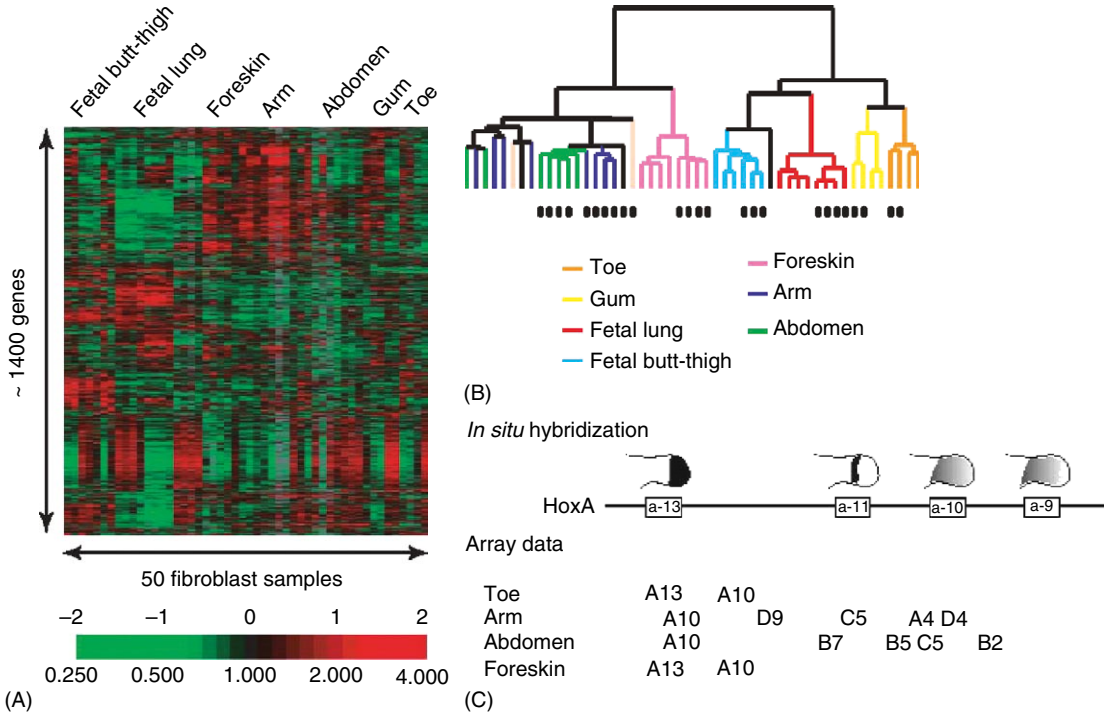


FIGURE 51-3 Topographic differentiation of fibroblasts identified by microarray analysis. (A) Heat map of fibroblast gene expression patterns. Fibroblasts from several anatomic sites were cultured, and their mRNAs were analyzed by cDNA microarray hybridization. Approximately 1400 genes varied by at least three-fold in two samples. The fibroblast samples were predominantly grouped together based on site of origin. (B) Supervised hierarchical clustering revealed the relationship of fibroblast cultures to one another. Site of origin is indicated by the color code, and high or low serum culture condition is indicated by the absence (high) or presence (low) of the black square below each branch. Because fibroblasts from the same site were grouped together irrespective of donor, passage number, or serum condition, topographic differentiation appeared to be the predominant source of gene expression variation among these cells. (C) *HOX* expression in adult fibroblasts recapitulates the embryonic Hox code. In a comparison of *HOX* expression pattern in secondary axes, schematic of expression domains of 5′ *HoxA* genes in the mouse limb bud at approximately 11.5 days post coitum is shown on top. The *HOX* genes up-regulated in fibroblasts from the indicated sites are shown below. *HoxC5* is expressed in embryonic chick forelimbs, and *HoxD9* functions in proximal forelimb morphogenesis. (*Discussed in detail in Chang et al.*, 2002.)

may be approached by microarray analysis. One approach to understanding stem niches is to explore the diversity of the normal tissue compartments, especially that provided by resident stromal cells.

An illustrative demonstration of this concept is a recent study of global gene expression patterns of fibroblasts derived from different anatomic sites of skin. Chang *et al*. cultured fibroblasts from multiple sites of human skin, and microarray analysis of their global gene expression patterns revealed that fibroblasts from different sites have distinct gene expression programs that have the stability and diversity characteristic of differentiated cell types (Figure 51-3). Some of the site-specific gene expression programs in fibroblasts included components of extracellular matrix and many cell fate signaling pathways, including members of transforming growth factor β, Wnt, receptor tyrosine kinase, and G-protein coupled receptor signaling pathways. An intriguing hint to the specification program in fibroblasts is the maintenance of key features of the embryonic Hox code (which specifies the anterior-posterior body plan) in adult fibroblasts. Thus, stromal cells such as fibroblasts are likely to encode position-specific information in a niche that specifies the developmental potential of interacting stem cell populations. In the case of fibroblasts, because their positional identities are maintained in vitro (as evidenced by the fidelity of the Hox expression patterns), it is likely that efforts using stem cells to develop artificial tissue and organs will benefit from incorporation of site-specific fibroblasts, or their molecular signatures that recreate the stem cell niche. Identification of the specific stem cell niches—and specific cell fate determining pathways—can thus be accelerated by a comprehensive description and understanding of the stromal cell diversity, using microarray analysis.

KEY WORDS

DNA microarray A collection of genes spotted on a solid surface (often a glass slide), arranged in rows and columns, so that the origin of each spot is known. Depending on the type of microarray, the spots consist of cDNA sequences amplified by PCR or synthetic oligonucleotides. Each DNA microarray may contain tens

of thousands of gene spots, and is therefore a powerful tool for the study of gene expression patterns on a genomic scale.

Hierarchical clustering A method of data-mining in which members of a data set are statistically grouped or clustered together according to a certain parameter (e.g., gene expression profile) in a way that maximizes intracluster similarity and minimizes intercluster similarity. In the clustering process, two clusters are merged only if the interconnectivity and closeness (proximity) between two clusters are high relative to the internal interconnectivity of the clusters and closeness of items within the clusters.

Supervised analysis Statistical methods to identify genes that are differentially expressed between groups of samples, as well as to find genes that can be used to accurately predict the characteristics of groups.

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Chapter 52

Zebrafish and Stem Cell Research

Emily K. Pugach, K. Rose Finley, and Leonard I. Zon

INTRODUCTION

The utility of the zebrafish system in vertebrate biology has sparked the burgeoning development of a new and noteworthy field. Biologists across the world are taking advantage of *Danio rerio* for investigations into organogenesis, disease, and development. The small size, ease of care, and rapid generation time are but a few of the many aspects that make the zebrafish a particularly useful model organism. In addition, the optical transparency of zebrafish embryos is especially suited for investigations into hematopoiesis and stem cell biology.

Perhaps one of the most significant features of the zebrafish system is the ability to conduct large-scale forward genetic screens in an organism with high genetic conservation to the human genome. Screening imparts the ability to address specific developmental processes without any prior knowledge of what genes may be involved. The Zebrafish Genome project performed by the Wellcome Trust Sanger Institute facilitates the positional cloning of genes uncovered via genetic screens in the zebrafish (www.sanger.ac.uk). With regard to stem cells, the zebrafish has been utilized largely to understand hematopoietic stem cells (HSCs). The success of the system makes it likely that stem cells from other organs will be explored in the zebrafish. This chapter will discuss attributes of zebrafish for investigations into hematopoiesis, strategies for conducting genetic screens in the fish, current research in the field, and finally, the future of the zebrafish system for investigations into stem cell biology.

THE ZEBRAFISH SYSTEM

Morphological and Embryonic Attributes

Zebrafish are relatively small (3 to 4 cm) and reach sexual maturity at two to three months. The fish mate year round, with females mating weekly. Because of the small size of the fish, facilities are able to maintain thousands of individuals in a compact laboratory environment. Few, if any, vertebrate model organisms allow for such population size or ease of care. Furthermore, females lay between 100 and 200 eggs per mating, permitting large-scale genetic screens as well as Mendelian approaches and analysis.

Of particular importance to the study of early blood development and hematopoietic stem cells is the unique embryonic morphology of the zebrafish. Zebrafish embryos develop externally from the one-cell stage and are transparent, permitting embryonic development to be readily viewed under a dissecting microscope. Circulation begins by 24 hours post-fertilization (hpf), and the number and morphology of blood cells may be identified under the microscope.

Blood Formation in the Zebrafish

The hematopoietic program in vertebrates begins with mesodermal patterning along the dorsal–ventral axis. During the process of mesoderm patterning, a subset of cells gives rise to a putative population termed "hemangioblasts." These cells are hypothesized to be a common precursor of blood and vascular lineages, due to their close physical association and common gene expression during early development. Unlike many other vertebrates that form primitive HSCs in extraembryonic yolk sac blood islands, the initial site of blood formation in zebrafish is the inner cell mass (ICM), an intraembryonic tissue. The zebrafish ICM tissue is analogous to the extraembryonic mammalian blood islands in its role as the primary site of primitive erythropoiesis.

As in all vertebrates, a shift in hematopoietic sites occurs in the zebrafish. The primitive wave of hematopoiesis in the ICM is followed by hematopoietic development in a subsequent intraembryonic site, the aorta-gonad-mesonephros (AGM), the region of the dorsal mesentery and the ventral wall of the aorta. Recent fate-mapping studies by Muriyama *et al.* have also elucidated an additional site of hematopoiesis, the caudal hematopoietic territory (CHT). By injecting

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a caged fluorescent dye into the zebrafish embryo at the one-cell stage, and later uncaging the dye using a laser targeted specifically to stem cells, it has been shown that uncaged cells in the AGM expressing HSC-specific markers like *c-myb* and CD41 appear later in the CHT. It is thought that the CHT mimics many of the functions of the mammalian placenta or fetal liver, prior to definitive hematopoiesis in the kidney and thymus where the same hematopoietic cells that seeded the CHT are later traced. While the kidney will become the primary site of hematopoiesis in the zebrafish, the primary sites of definitive hematopoiesis in mammals are the fetal liver and the adult bone marrow. Despite some other differences (for example, fish erythrocytes remain nucleated), zebrafish blood is remarkably similar to mammalian blood, with all lineages—erythroid, myeloid, lymphoid, and thrombocytic—represented. Thus, the fish is a capable and applicable organism for the study of hematopoiesis.

Vogeli *et al.* also used the fate-mapping technique that was used to identify the CHT as a site of hematopoiesis to address the hypothesis of the hemangioblast, a common origin for blood and vascular cells. In order to monitor single cells, a caged fluorescent dye was again utilized in the zebrafish, and it was shown that single cells along the entire extent of the ventral mesoderm can give rise to both hematopoietic and endothelial cells. Thus, fate-mapping in zebrafish hematopoiesis lends support to the putative hemangioblast.

The identification and isolation of zebrafish homologs of factors deemed critical for normal hematopoiesis and vasculogenesis, such as transcription factors *scl*, *lmo2*, *GATA1*, *GATA2*, *c-myb*, *Fli1*, and *runx1* have permitted investigations into many aspects of blood and vascular tissue formation. RNA *in situ* hybridization studies using whole zebrafish embryos provide gene expression patterns that may by used to investigate the timing and anatomy of hematopoiesis and vasculogenesis (Figure 52-1). The first appearance of vascular and blood markers is seen at the three-somite stage (11 hpf) with the expression of *GATA-2* and *scl* in two stripes of lateral plate mesoderm. Some cells within these stripes will become blood in the fish. By the 18-somite stage (18 hpf), these expression stripes have fused to form the tubular ICM structure. The ICM is fully formed by 23 hpf, just before the commencement of circulation. The presence of *c-myb*- and *runx1*-expressing cells located in the ventral wall of the dorsal aorta at 36 hpf suggests the presence of definitive HSCs in the zebrafish. By two days postfertilization (dpf), HSCs have migrated to the developing kidney and thymus, where hematopoiesis will continue throughout the life of the fish.

Although the discrete steps necessary for the induction of hematopoietic precursors into differentiated blood lineages, as well as the program directing the different hematopoietic waves, are still not entirely clear, the zebrafish is a powerful model organism capable of further unraveling the story. It is evident that the process of differentiation of

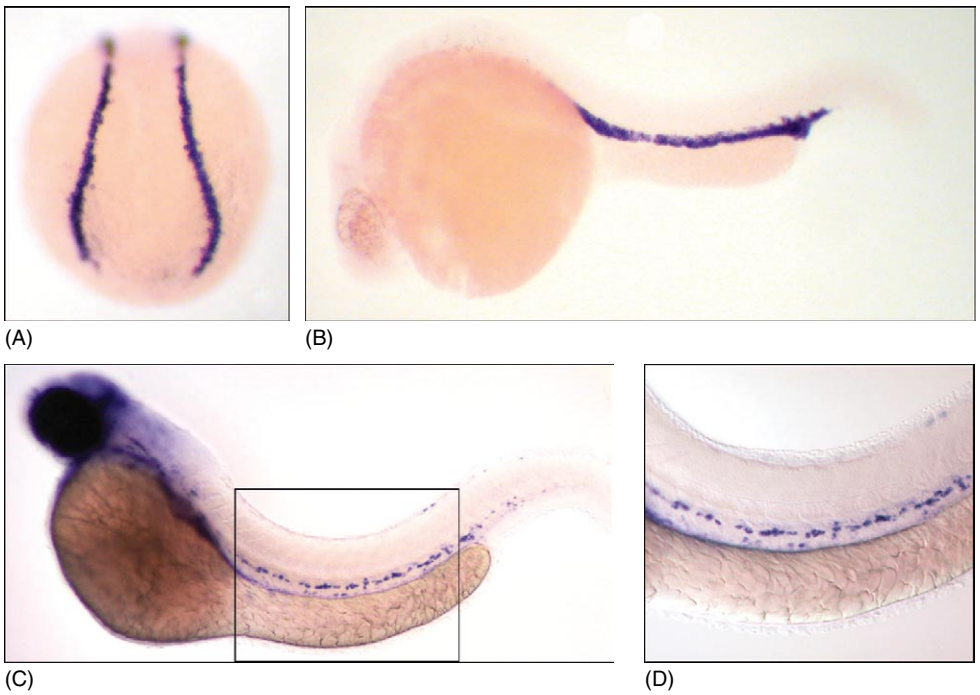


FIGURE 52.1 Expression patterns of specific hematopoietic factors in zebrafish embryos. (A) Whole embryo *in situ* hybridization for *scl* at 12 hpf shows the stripes of ventral mesoderm and marks putative hemangioblasts. (B) *GATA-1* expression in an embryo at 24 hpf marks the ICM prior to circulation of erythroblasts. (C) *c-myb* at 36 hpf marks the wall of the dorsal aorta and the zebrafish equivalent of the mammalian AGM. Shown enlarged in (D). (Courtesy of C. E. Burns).

hematopoietic stem cells into the erythroid, myeloid, and lymphoid lineages is the result of a highly-conserved gene program. It is also clear that the zebrafish, like other vertebrates, possesses discrete waves of hematopoietic events, characterized by expression of specific and recognized transcription factors. As will be discussed below, zebrafish have played an important role in uncovering novel genes involved in these programs via large-scale genetic screens.

GENETIC SCREENS IN ZEBRAFISH

The first large-scale genetic screens in a vertebrate organism were conducted in zebrafish in Boston, Massachusetts, and in Tübingen, Germany, in the mid-1990s. The ability to conduct screens in a vertebrate organism, in addition to those conducted in such nonvertebrates as *Caenorhabditis elegans* and *Drosophila melanogaster*, is clearly an important achievement. Such screens have already yielded scores of mutants, with defects in varying stages of blood and vasculature formation, a few of which will be discussed in detail in this chapter. The power of screens in zebrafish is the ability to uncover novel genes involved in specific developmental processes in an unbiased manner.

The Boston and Tübingen ventures utilized F_2 screens, in order to uncover developmental mutants (Figure 52-2). The screens began with the mating of a male fish that had been treated with the chemical ethylnitrosourea (ENU) to a wild-type female. Mutations present in the germ line of the ENU-treated male were passed to heterozygous individuals in the F_1 generation. The F_1 and F_2 generations were successively incrossed to obtain an F_3 generation. In the presence of a recessive mutation, half of an F_2 family should be heterozygous for that mutation. When two F_2 are mated together and both are heterozygous for the mutation, their progeny will display 25% wild-type, 50% heterozygous, and 25% homozygous mutant embryos. The Boston and Tübingen screens began with approximately 300 ENU-treated males, and uncovered more than 2000 new developmental mutants.

An important aspect of the zebrafish is the ease with which mutations may be generated in the fish. The chemical ethylnitrosourea (ENU), which causes point mutations throughout much of the genome, is a popular and effective mutagen for zebrafish. However, many other chemicals and avenues are available to achieve mutations in the fish, all achieving slightly different results. Gamma and X-rays may be utilized to create large genomic deletions and translocation events, as well as point mutations. In a newer approach, insertional mutagenesis has been employed in zebrafish. Mutations are achieved by injecting retroviruses into the 1000–2000 cell-stage embryo. Although the degree of mutations achieved by this approach is significantly lower than ENU mutagenesis, the ease of cloning the mutated gene makes it an exciting resource for the field.

In addition to the F_2 diploid screens conducted in Tübingen and Boston, haploid and gynogenetic diploid screens have also been designed and carried out in zebrafish. Zebrafish embryos are able to survive three days postfertilization as haploid organisms. This allows a shortcut to uncovering mutations, as recessive alleles in F_1 fish may be uncovered in a single generation, as opposed to the two generations needed in diploid screens. To create viable haploid embryos, a female fish is gently squeezed to release her eggs. Sperm from male fish is harvested from testes and UV-inactivated before adding to the unfertilized eggs. Because the UV irradiated sperm is unable to contribute any genetic material to the eggs, the embryos develop with only the genetic material of the mother. Haploid embryos have the same body plan as diploids; however, they are shorter in length and possess some developmental problems, as well as a lifespan of only three to five days. Nonetheless, haploid screens have successfully uncovered many types of developmental mutations, and have been proven to be an extremely significant resource.

In addition to haploid embryos, gynogenetic embryos may also be utilized in zebrafish screens. By applying hydrostatic pressure or heat shock, unfertilized eggs from female fish can be forced to become diploid, comprised solely of the mother's genetic material. Like haploid



FIGURE 52.2 GATA-1 promoter driving GFP in transgenic zebrafish embryo at 24 hpf. (A) The transgenic embryo as seen under dissecting scope. (B) Under fluorescence the GATA-1 driving GFP expression localizes to the ICM. (GATA-1 GFP line courtesy of S. Lin; Photos courtesy of R. Wingert.)

embryos, gynogenetic diploid embryos represent the genome of one female fish, and the large size of the female's clutch allows the implementation of Mendelian genetics. Gynogenetic diploid embryos will survive to adulthood, unlike haploid embryos, and will allow screening for later developmental abnormalities in the fish. This method bypasses the lethal haploinsufficiency of haploid embryos, while maintaining identical genomic information. With the use of haploid and gynogenetic embryos, the genome of a female fish may be screened for recessive mutations displaying a phenotype in her progeny. These approaches provide a less burdensome method for uncovering mutations involved in specific developmental pathways.

ZEBRAFISH BLOOD MUTANTS

Many blood mutants have been uncovered in large-scale screens, and several others have occurred spontaneously, or been discovered while screening for mutations in other developmental areas. Characterization and attempts to clone these genes have provided valuable information into hematopoiesis and its genetic program. Some mutants have also proved useful as new models for human diseases. The blood mutants have been divided into discrete categories, based on their stage of disruption in the hematopoietic scheme. Categories of blood mutants include mesoderm patterning mutants, hematopoietic stem cell mutants, committed progenitor mutants, proliferation and/or maintenance mutants, hypochromic mutants, and photosensitive mutants. Mutants involved in the first three of these categories will be discussed in this section.

The zebrafish mutant dubbed *spadetail* (*spt*) is in the category of mesoderm patterning. The *spt* gene was shown to be a T-box transcription factor, necessary for correct release from mesoderm-inducing signals as well as proper formation of endoderm. The phenotype of *spt* is one of incorrect trunk formation, poorly-formed dorsal somites, undifferentiated blood, disorganized dorsal vessel formation, and other characteristics, indicating severe mesoderm and endoderm tissue deficiencies.

Other mesoderm patterning mutants have been identified in zebrafish, including *swirl* (*swr*) and *snailhouse* (*snh*), which have been shown to have increased dorsal tissue, leading to scarcity of ventral blood. Both *swr* and *snh* phenotypes are caused by mutations in members of the bone morphogenetic protein (BMP) subgroup belonging to the TGF-β superfamily of growth factors. BMP signaling is critical for correct mesodermal patterning along the ventral axis, and plays a key role in the antagonistic relationship between dorsalizing and ventralizing signals during mesoderm patterning and ultimately the correct formation of blood. Whereas these mutants are not blood mutants directly, *spadetail*, *swirl* and *snailhouse* are prime examples of the importance of correct patterning for both proper blood and proper vessel formation.

Another recently-discovered mutant in the BMP signaling pathway, *lost-a-fin*, is a mutant of the type I BMP-receptor, *alk8*. Found in a screen by Hogan *et al.* (2006) for early myeloid failure, the *alk8* mutant ties BMP signaling to early myeloid specification. Erythrocytes are predominantly generated in the posterior region of the fish, while the generation of the myeloid population takes place in a physically-separate region in the anterior part of the fish. Although some genes, including *GATA-1* and *pu.1*, are known to contribute to this spatial separation of these separate hematopoietic events, the type I BMP-receptor has now been shown to be required for *pu.1* expression and myelopoiesis. Thus, the *alk8* mutant reveals a novel factor in the differential induction of anterior and posterior hematopoiesis.

The *kugelig* (*kgg*) mutant, revealed because of its abnormal anteroposterior patterning, has an early defect in hematopoiesis in which blood progenitors are not specified. The responsible mutation has been mapped to the caudal-related homeobox gene, *cdx4*. By demonstrating that *kgg* mutants also have irregular *hox* gene expression, it has been determined that *cdx4* is a transcription factor that plays a role in the regulation of the *hox* gene cluster. By overexpressing certain *hox* genes, such as *hoxa9a*, the *kgg* blood phenotype can be partially rescued, thereby demonstrating the combined role of *cdx4* and the *hox* genes in the development of the hematopoietic system.

Another interesting blood mutant is *cloche* (*clo*), which has been shown to possess an almost complete absence of blood and vasculature in studies, as well as reduced expression of transcription factors necessary for both blood and vessel formation. The abnormally low expression of *scl*, *lmo2*, *GATA-2*, and *GATA-1* suggest that *clo* has a loss of blood progenitors. In addition, *clo* embryos have severely reduced expression of *flk1* or *tie1*, both shown to be expressed by blood and endothelial progenitors.

The almost complete lack of blood and vasculature in *cloche* suggests that the *clo* gene product may be responsible for maintenance or differentiation of the putative hemangioblast. Interestingly, overexpression of *scl* has been shown to partially rescue *GATA-1*, *flk1*, and *tie1* expression in *clo*, but it has been ruled out as a candidate due to its linkage group position. The *cloche* gene has been shown to act upstream of *flk1* in the program of endothelial cell differentiation. The *clo* mutant demonstrates the ability of genetic screens to uncover novel genes associated with stem cell biology.

The *bloodless* (*bls*) mutant, like *clo*, may be placed in the category of hematopoietic stem cell mutants. Embryos carrying the *bls* mutation have been shown to possess almost no erythroid cells at the onset of circulation, but contain normal macrophages and carry out normal, though delayed, lymphopoiesis. Although dominant, the *bls* mutant is not totally penetrant in its display of an almost complete lack of primitive erythrocytes. Corresponding with the lack of embryonic erythroid cells are decreased levels of *scl* and

GATA-1 expression in *bls*. Levels of *GATA-2* expression are also reduced but present in *bls* mutants, likely due to the presence of *GATA-2* expressing endothelial precursors in the ICM. The *bls* mutant does in fact possess normal vessel formation, and has normal *flk1* expression during embryogenesis. Interestingly, *bls* embryos produce normal red blood cells at approximately 5 dpf, after the initiation of the definitive wave of hematopoiesis. These results suggest that the *bls* gene product is responsible for maintenance or production of hematopoietic precursors during primitive hematopoiesis, but does not affect production of HSCs. This mutant should provide much insight into the method of differentiation of stem cells from precursors, and for the connection between primitive and definitive waves of embryonic hematopoiesis.

The *moonshine* (*mon*) mutant not only displays virtually no circulating red blood cells in early embryogenesis, but unlike *bls*, was shown by Ransom *et al.* (1996) to fail to develop erythrocytes throughout the lifespan of the fish. *GATA-1* expression is initiated normally in *mon* fish, indicating that the commitment of erythroid cells is not affected by the mutation. However, *GATA-1* expression is gradually lost during development, and committed erythroid cells undergo major apoptosis. Positional cloning has shown that *mon* encodes the zebrafish ortholog of mammalian transcriptional intermediary factor 1gamma (TIF1gamma), a member of the TIF1 family of transcriptional cofactors. The cloning of this gene in zebrafish establishes a significant role for this transcriptional intermediary factor in the differentiation of hematopoietic cells in primitive and definitive erythropoiesis in vertebrates.

In a study performed by Burns *et al.* (2005) one of the major molecular pathways regulating hematopoietic stem cell expansion in the zebrafish was identified by examining Notch signaling in the mutant, *mindbomb*. In *mindbomb* fish that lack Notch signaling, expression of both *c-myb* and *Runx1* in the AGM is severely reduced compared to wild-type animals. However, overexpression of *Runx1* rescues the diminished embryonic HSC phenotype. This establishes a novel relationship between *Runx1* and Notch signaling in developmental specification of HSCs. In addition, Notch induction in irradiated adult zebrafish was shown to accelerate multi-lineage hematopoietic cell repopulation in the kidney marrow, thereby elucidating the role of the Notch-Runx pathway in the homeostasis of HSC number. Hence, the *mindbomb* mutant has proved to be a useful tool in elucidating the role of the Notch-Runx pathway in the emergence of HSCs in the AGM, as well as in their maintenance of the hematopoietic system of the mature fish.

CELL SORTING AND TRANSPLANTATION

As in other vertebrate models, differentiated blood cell populations in the zebrafish can be sorted via flow cytometry. Samples collected from the kidney, spleen, and blood can be analyzed by their light scatter characteristics. Further, flow cytometry of kidney tissue, the site of adult definitive hematopoiesis, reveals distinct scatter populations.

Scatter populations of kidney tissue correspond to the major blood lineages, and can be used to isolate pure populations of mature erythroid cells, myelomonocytic cells, lymphoid cells, and immature precursors. The percentage of cells in each category obtained by morphological cell counts in normal adults corresponds to the percentage of each cell type found via flow cytometry. Thus, the use of scatter profiles to evaluate and quantify discrete populations of differentiated cells is an accurate and important tool in both adult wild-type and mutant zebrafish.

Flow cytometry analysis may be used to characterize the blood mutants obtained through genetic screens in the fish. The light scatter populations of mutant fish may be compared to those of wild-type fish, enabling the quantification of population deficits or increases in specific blood lineages in the mutant fish. Many primitive blood mutants exhibit a homozygous lethal phenotype, making analysis of adult homozygous mutants impossible. The analysis of blood lineages of adult heterozygous fish carrying only one copy of the mutant allele, however, can be revealed using this technique. This technique has exposed several cases of haploinsufficiency, suggesting that many genes required for embryonic and primitive hematopoiesis are additionally important in the adult fish.

The technique of flow cytometry also enables the collection and purification of GFP-expressing cells present in zebrafish transgenic lines. The creation of transgenic lines has become common in zebrafish studies, as it allows visualization of specific tissues in the transparent embryo (Figure 52-2). GFP lines have been created with the *GATA-1* promoter to produce fluorescent erythroid cells and the *lmo2* promoter creating zebrafish with fluorescent green blood and vasculature. GFP+ cells in these transgenic fish can be isolated from the rest of the population of cells in the fish using flow cytometry.

Once isolated, purified blood lineages may be collected and transplanted into recipient fish. Transplantation assays may be used to measure many different aspects of hematopoiesis in mutant and nonmutant fish alike. In one assay, hematopoietic stem cell activity in mutants is characterized by injecting kidney cells harvested from lines of fish in which the *GATA-1* promoter drives GFP. The donor transgenic HSCs will produce GFP+ erythrocytes, which are continuously made and replenished from upstream stem and progenitor cells. Many transplant recipients possess GFP+ cells up to six months post-transplantation, indicating the existence of a long-term repopulating stem cell in the zebrafish kidney.

Recently, it has been shown by Traver *et al.* (2002) that the hematopoietic system in the zebrafish can be ablated by gamma irradiation. This finding, combined with the existence

of a long-term repopulating stem cell in the zebrafish, further enhances the field of transplantation assays. Hematopoietic stem cell transplants can rescue irradiated fish, and modifications of this assay can be used to study HSC function or enrichment strategies.

Another new tool for the zebrafish transplant field is the transparent adult zebrafish, *casper*. A double-homozygous mutant of *nacre*, the gene encoding *mitfa*, and *roy orbison* (gene unknown), *casper* lacks both melanocytes and iridophores, so that much of the adult fish's internal anatomy is easily visible using standard stereomicroscopy. Further, the use of confocal microscopy allows the resolution of a single cell. Such optical clarity in adult fish allows for an even greater understanding of hematopoietic stem cell activity following transplants than is provided by standard FACS analysis. When fluorescently-labeled hematopoietic stem cells are transplanted into irradiated *casper* recipient fish, hematopoietic progenitor engraftment can be readily visualized from within mere hours following the initial transplant to as many as five weeks post-transplantation. In as little as two weeks, fluorescent populations of cells can be visualized within the region of the zebrafish kidney and in the vasculature, using only a standard fluorescent stereomicroscope. Such a tool in the zebrafish transplant field represents a unique opportunity to monitor the *in vivo* dynamics, in real-time, of hematopoietic stem cell transplants, as well as metastasis of disease.

DISEASE MODELS IN ZEBRAFISH

Zebrafish mutants have been shown to resemble human diseases including hematopoietic, cardiovascular, and kidney disorders. Although other organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* have been extensively studied with respect to developmental processes and may be used in screens, they do not address many of the morphological aspects unique to vertebrates. The zebrafish, however, is a vertebrate system and thus may be used to address developmental processes in the kidney, in multi-lineage hematopoiesis, and in notochord as well as neural crest cells, among others. Zebrafish mutants have played an important role not only in clarifying vertebrate-specific developmental processes, but also as models for specific human disorders.

The hematopoietic mutant known as *sauternes* (*sau*) exhibits delayed maturation of erythroid cells and abnormal globin expression. The *sau* mutant fish develops a microcytic hypochromic anemia. Cloning of the *sau* gene revealed the gene product to be aminoleulinate synthase (ALAS2), an enzyme required for the first step of heme biosynthesis. Mirroring the fish genetic disease, humans with a mutation in ALAS2 gene have been found in a study by Edgar *et al.* to have congenital sideroblastic anemias. *Sau* is the first animal model of this disease.

The zebrafish mutant known as *retsina* (*ret*) has recently been shown by Paw *et al.* to exhibit an erythroid-specific mitotic defect with dyserythropoiesis comparable to human congenital dyserythropoietic anemia. This work identifies a gene (*slc4a1*) encoding an erythroid-specific cytoskeletal protein necessary for correct mitotic divisions of erythroid cells. In addition to modeling a human disease, this study demonstrates the notion of cell-specific mitotic adaptation.

Positional cloning of the zebrafish mutant *wiessherbst* identified the gene *ferroportin1*, a transmembrane protein responsible for the transport of iron from maternal yolk stores into circulation, solving the puzzle of the elusive iron exporter. This gene was first identified in zebrafish. Later research conducted by Njajou *et al.* determined that humans with mutations in *ferroportin1* develop the human disease type IV hemachromatosis. A small peptide called hepcidin binds to ferroportin and regulates iron export in a variety of tissues. This peptide may be an important modulator of the anemia of chronic disease.

Transgenic zebrafish have also been created to study oncogenic disease. In one such investigation, Langenau *et al.* created a model for T-cell leukemia in the fish, using the lymphoid-specific *rag2* promoter driving mouse *c-myc*, a gene known to function in the pathogenesis of leukemias and lymphomas. In addition, a chimeric transgene consisting of *myc* fused to GFP allowed the visualization of the spread of tumors from the thymus of affected fish. Tumors in these transgenic fish from this study may now be used for suppressor/enhancer genetic screens, in order to identify components either lessening or worsening the leukemic progress. In addition to this model of T-cell leukemia, models in the zebrafish have been created for many other cancers, including rhabdomyosarcoma, melanoma, and pancreatic cancer. These transgenic fish can also be used in chemical screens to identify novel therapeutic agents to treat disease.

Further studies are being conducted in zebrafish to tie mutant phenotypes to known, as well as undocumented, human disorders. The use of the zebrafish system to gain understanding of human disease states has been proven, and is evolving as technologies advance. Screens continue to uncover mutant phenotypes with specific developmental abnormalities, many of which will play a role in deciphering human disease states. Models of disease in zebrafish may be used as tools for drug-target and pharmaceutics discovery in large-scale chemical screens.

THE FUTURE OF THE SYSTEM

In a recent study, North *et al.* (2007) have defined a specific role of the prostaglandins in the production and regulation of HSC homeostasis in the AGM. Revealed by a chemical genetic screen aimed at identifying novel modulators of HSCs, prostaglandin E2 (PGE2) increases stem cell production in the AGM in treated embryos. Conversely,

the reduction of PGE2 effectively reduces HSC numbers. Treatment of irradiated adult fish with PGE2 affects the homeostasis of definitive adult hematopoiesis, successfully enhancing post-irradiation hematopoietic recovery. Further, PGE2 has been shown to have the same effect in the mouse model, indicating a conserved PGE2 pathway throughout the vertebrate system, and thereby demonstrating the capacity of the zebrafish in the discovery of novel drugs.

The study of genetics also continues to be a useful and exciting field in the zebrafish. Although genetic screens in the zebrafish were once restricted to forward genetic approaches, new methods in reverse genetics now allow researchers to examine the functional dynamics of specific genes of interest in many different ways. Transiently, a gene of choice may be overexpressed by the injection of mRNA of the gene into the fish embryos. Alternatively, a gene may be knocked-down temporarily by injecting a morpholino oligonucleotide targeted to the gene of interest. While these techniques allow the study of gene function in transience, the technique of injecting one-cell stage embryos with a transgene of choice can produce stable transgenic fish whose lines can be propagated. Further, the introduction of TILLING (Targeted Induced Local Lesions In Genomes) in the zebrafish has allowed the use of directed mutagenesis. This approach was used by Berghmans *et al.* (2005) to create the first *TP53* mutant fish lines. As this tumor-suppressor gene is mutated in nearly 50% of all tumors, it is an especially useful model for the study of oncogenic disease. Already it has been shown that homozygous *TP53* mutant fish develop tumors, particularly malignant peripheral neural sheath tumors, and that cooperation with other mutations can accelerate onset and growth of these and other types of tumors.

Another body of work has focused on zebrafish tail regeneration. The zebrafish is able to regenerate fins, a unique characteristic of some lower vertebrates. Little is known about the molecular and cellular processes involved in regeneration, and genetic screens have been, and are continuing to be, employed to identify essential genes. The process of differentiation, patterning, and proliferation, as well as the existence of pluripotent cells in adult tissues, are being examined in zebrafish, and remain extremely compelling for the field of stem cell biology. Although research is still at an early stage, regeneration studies may also address human tissue repair, stem cell transplantation, and even tissue-engineering.

Pioneering studies have been, and are currently being, conducted that reveal an exciting future for the zebrafish system. Large-scale genetic and chemical screens, cell-sorting techniques, and transgenic technologies have been utilized in elegant investigations into hematopoiesis and stem cell biology. Further dissection of developmental processes and their connections to human disease will surely benefit from the utility of this model organism.

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KEY WORDS

Gynogenesis Manipulation of ploidy by suppression of the polar body in an egg lacking paternal genetic contribution.

Hemangioblast The postulated bipotential cell that would give rise to hematopoietic and endothelial cell lineages in vertebrates.

Hematopoiesis The formation and development of blood cells.

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Part V

Applications

Chapter 53

Cancer Stem Cells

Michael Rothenberg and Michael F. Clarke

The worldwide burden of cancer is immense, yet our ability to cure most cancers sadly remains quite limited. Tumors are clonal, heterogeneous collections of abnormal cells whose development is only partially understood. Recently, the Cancer Stem Cell (CSC) model has re-emerged as a compelling way to reconcile the seemingly contradictory properties of clonality and developmental heterogeneity by postulating that a subset of cells within certain tumors—the cancer stem cells—self-renew, give rise to various differentiated tumor cells, and ultimately drive tumor growth and metastasis. The modern tools of stem cell biology have provided strong evidence to support this model—particularly with the prospective isolation of CSCs from hematologic and solid malignancies in both mice and humans. As molecular pathways underlying self-renewal have been dissected, the potential for cancer stem cells to arise from both normal stem cells as well as from non self-renewing progenitor cells has become apparent. The term "cancer stem cell" is thus an operative definition that does not imply a relationship to a normal tissue stem cell. Ultimately, cancer stem cells will likely have profound effects on our conceptualization and treatment of cancer.

INTRODUCTION

Cancer biologists have long recognized that tumors are complex collections of aberrant cells, each of which ultimately derives from a single ancestor, the cell of origin. The process by which the cell of origin develops into an ever-expanding, heterogeneous tumor that retains many features of its parent tissue remains mysterious. Recently, the cancer stem cell hypothesis has re-emerged as a compelling model to explain both the clonality and some of the heterogeneity of tumors. It postulates that each tumor contains a subset of cells—the cancer stem cells—that are uniquely responsible for tumor growth, heterogeneity, and metastasis. These specialized cells possess two key features that together distinguish them from the remainder of the tumor cells: self-renewal and differentiation potential. Substantial evidence from clinical oncology, as well as from experiments on human and animal tumors, provides strong support for this model. Most importantly, cancer stem cells have now been prospectively identified from leukemias and many different solid tumors in mice and humans. Simultaneously, significant progress in stem cell biology has provided insights into the molecular mechanisms by which normal stem cells self-renew. Interestingly, experiments on non-self-renewing cells have demonstrated that self-renewal is a property that cells can acquire through mutations. This finding sheds light on the possible origins of cancer stem cells, and illustrates how they may originate from either self-renewing or non self-renewing cells. The existence of cancer stem cells has important implications for how we conceptualize and treat cancer. This review provides an overview of the cancer stem cell model, and the data that support it.

CLONALITY AND HETEROGENEITY IN TUMORS

Tumors Have a Single Cell of Origin

The clonality of tumors is a fundamental principle in cancer biology. Each tumor—no matter how large or heterogeneous—ultimately originated from a single transformed cell, called the cell of origin. This principle was first demonstrated in malignancies in women, using the phenomenon of X chromosome inactivation (Fialkow, 1972; Fialkow *et al.*, 1972; Gale and Linch, 1998). During early female development, the process of lyonization causes different somatic cells (and their progeny) to randomly inactivate one of their two X-chromosomes; consequently, an adult woman is a mosaic of two distinct cell populations, expressing either the maternal or the paternal X-chromosome. When tumors from women who are heterozygous for various X-linked loci are characterized, the cancerous cells exhibit only one pattern of X-chromosome inactivation, suggesting a common (clonal) origin. Other analyses using the highly polymorphic X-linked HUMARA (human androgen receptor) locus, as well as distinct stably-inherited patterns of promoter methylation on different X-chromosomes, have been

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used to further demonstrate this point (Fearon *et al.*, 1987; Vogelstein *et al.*, 1987).

Tumor clonality can also be demonstrated for malignancies in which all the cancerous cells harbor the same chromosomal translocation, such as the Philadelphia chromosome (Yoffe *et al.*, 1987). Similarly, lymphoma cells that all harbor the same genomic rearrangements at the immunoglobulin locus or the T-cell receptor locus must have originated from a common origin. Although a few studies have suggested that tumors may not always be clonal in their earliest stages (Novelli *et al.*, 1996; Thliveris *et al.*, 2005; Halberg and Dove, 2007), the clonality of cancer has emerged as a central principle in tumorigenesis. This idea also has held true for other solid tumors using similar techniques.

Tumors Resemble "Aberrant Organs"

In the context of this clonality, the cancer cells of all but the most highly aggressive tumors appear heterogeneous. This is readily apparent on routine hematoxylin and eosin (H&E) staining, which shows malignant cells in varying stages of differentiation. For example, cancers originating in glandular epithelial tissues (adenocarcinomas) often elaborate rudimentary glands with distinct basolateral and luminal sides, which may even retain the ability to generate tissue-specific secretions: milk proteins in the case of breast cancers; prostatic secretions in the case of prostate cancers; and mucus in the case of colon cancers. Likewise, cancers originating in squamous epithelia (squamous cell carcinomas) form keratinous pearls, suggestive of their tissue of origin. An extreme example of tumor heterogeneity is a teratoma, which contains immature (embryonic carcinoma) cells, as well as differentiated cancerous cells from all three germ layers: endoderm; mesoderm; and ectoderm. On the other side of the spectrum is Burkitt's lymphoma, a highly aggressive, rapidly-dividing B-cell malignancy, characterized by homogeneous sheets of pleiotropic cells.

Not unexpectedly, phenotypic heterogeneity is also evident on analysis of gene expression (for example, by *in situ* hybridization) and immunohistochemistry. Various genes and proteins are specifically and differentially expressed in subsets of tumor cells. Sub-populations within a tumor may also differ from each other in other attributes: their ability to migrate or invade; their proclivity to enter the cell cycle or undergo apoptosis; their responsiveness to drugs or hormones; their ability to exclude vital dyes; their karyotype; and their ability to sustain growth in culture (Scharenberg *et al.*, 2002; Hirschmann-Jax *et al.*, 2004; Singh *et al.*, 2004) This heterogeneity and organization suggests that the development of a tumor from a single cell may recapitulate a developmental program. Thus, tumors can often be seen as caricatures of normal tissue or "aberrant organs," an idea that can be traced back to Rudolf Virchow, the father of modern cellular pathology (Virchow, 1858; Fidler and Hart, 1982; Heppner, 1984; Reya *et al.*, 2001; Lobo *et al.*, 2007).

THE CANCER STEM CELL MODEL

Models to Explain Both Clonality and Heterogeneity

Since tumors are both clonal and heterogeneous, a key question emerges: which cells drive the growth of a tumor? For example, is the property of tumorigenicity passed on from the cell of origin to all progeny, or is tumorigenicity a property possessed by only a subset of tumor cells? This question is relevant not only to understanding the basic biology of cancer, but also to determining the most effective treatment strategy in patients. If all cells in a tumor are potentially tumorigenic, then any curative treatment must eliminate all malignant cells. However, if only a fraction of the cells drive tumorigenesis, then a treatment that eliminates this sub-population while sparing some of the other cancer cells could result in long-term remission in at least some patients (Reya *et al.*, 2001; Al-Hajj *et al.*, 2004).

At least two different models attempt to explain both clonality and tumor heterogeneity: the stochastic model; and the cancer stem cell model. The stochastic model is based on the notion that the transforming events that endow the cell of origin with a malignant phenotype cause all progeny of that cell also to be tumorigenic. In this model, tumor heterogeneity is caused by subclones of tumor cells that result from a combination of different microenvironments and random genetic changes (including point mutations, chromosomal rearrangements, aneuploidy, and gene amplifications) (Nowell, 1976; Lengauer *et al.*, 1998). Ultimately, this model implies that effective treatment for cancer must involve eradication of all cancer cells.

The cancer stem cell (CSC) model postulates that only some tumor cells are tumorigenic—the cancer stem cells. When they divide, these malignant cells self-renew, and also give rise to the non-self-renewing cells that go on to elaborate the heterogeneous cells within a cancer. In this way, cancer stem cells both drive tumor growth, and also initiate the execution of a developmental program (Virchow, 1858; Fialkow, 1972; Mintz and Illmensee, 1975; Illmensee and Mintz, 1976; Fialkow, 1990). Cancer stem cells thus utilize pre-existing developmental hierarchies, and give rise to some of the cellular heterogeneity seen in such tumors.

Cancer Stem Cell, a Functional Definition

Importantly, the term "cancer stem cell" is an operative definition that does not necessarily connote a developmental relationship to normal stem cells; instead, it merely

states that a subset of cells within a tumor can self-renew and elaborate tumor heterogeneity (Clarke *et al.*, 2006). Ultimately, the definition depends on the assays of self-renewal and tumorigenicity. Also, the cancer stem cell model does not imply what percentage of cells within a tumor are cancer stem cells. Some very aggressive tumors may have a high percentage of cancer stem cells (Kelly *et al.*, 2007; Kennedy *et al.*, 2007), and chemotherapeutic treatments may increase the frequency of cancer stem cells in a tumor (Dylla *et al.*, 2008); however, neither of these observations affects the operative definition.

The stochastic model and the cancer stem cell model are not mutually exclusive. Since they are malignant cells, cancer stem cells likely exhibit genomic instability, and have a mutator phenotype (Lengauer *et al.*, 1998; Hanahan and Weinberg, 2000). Thus, as they divide and give rise to both tumorigenic and non-tumorigenic progeny, subclones of cancer stem cells may develop. Thus, the cancer stem cell population within a tumor may become heterogeneous (Odoux *et al.*, 2008). Also, different subclones of cancer stem cells may give rise to genomically distinct non-tumorigenic cells.

DEFINING FEATURES OF STEM CELLS IN NORMAL TISSUES

Self-renewal and Differentiation

Tissues are complex, organized collections of cells that conduct specialized functions using multiple distinct terminally-differentiated effector cells. Often, these effector cells have lifespans that are only a fraction of the lifespan of the organism. Thus, in order to maintain tissue homeostasis, and prevent a tissue from degenerating, tissues must have a mechanism for self-renewal; by necessity, this involves cellular proliferation and differentiation. This is accomplished by multipotent tissue stem cells, which are developmentally committed, but relatively undifferentiated, cells that can give rise to one or more differentiated cell types (Figure 53-1).

Stem cells have two features that together distinguish them from the other cells in a tissue: self-renewal; and differentiation potential. Self-renewal is the ability of a cell to divide and give rise to at least one daughter cell that has extensive proliferative capacity and the same developmental potential as its parent (Weissman, 2000; Melton D, 2004). This feature distinguishes stem cells from the other cells in the tissue, including progenitor cells. The rigorous experimental demonstration of long-term self-renewal can be challenging, since the developmental potential of a cell may not become fully evident until several cell divisions have elapsed (Weissman *et al.*, 2001).

Differentiation is the ability of a committed cell to give rise to one or more developmentally-distinct mature cell types. This is a property that is not entirely unique to stem cells. For example, both hematopoietic stem cells and progenitor cells can give rise to all the major hematopoietic lineages (Figure 53-1). However, because only the stem cells self-renew, if the bone marrow of a lethally-irradiated mouse is reconstituted with progenitor cells rather than stem cells, adequate multi-lineage hematopoiesis will only persist for a finite period of time (Spangrude *et al.*, 1988; Morrison *et al.*, 1995). Eventually, the progenitor cells will stop contributing to hematopoiesis, and the mouse will succumb (in approximately eight weeks) as the terminally differentiated blood cells are exhausted.

The size of the stem cell compartment, i.e., the number of stem cells within a tissue at any given time, is under tight genetic control (Muller-Sieburg and Riblet, 1996; Muller-Sieburg *et al.*, 2000). When a stem cell divides symmetrically to give rise to two stem cells, the stem cell compartment is expanded. Alternatively, when the stem cell divides asymmetrically to give rise to a stem cell and a non-stem cell, the stem cell compartment is maintained (Horvitz and Herskowitz, 1992; Morrison and Kimble, 2006). Of course, the stem cell compartment can also shrink if a stem cell divides to give rise to two non-stem cells. Other mechanisms for regulating the size of the stem cell compartment may also be at play. This probably has relevance to cancer biology, for one prediction of the cancer stem cell model would be that tumor suppressor genes might restrict the size of the cancer stem cell compartment. Also, more aggressive tumors may have a more pronounced cancer stem cell compartment (Dylla *et al.*, 2008).

Prospective Isolation of Normal Tissue Stem Cells

Adult tissue self-renewal has been best studied in the hematopoietic system, where hematopoietic stem cells (HSCs) have been unequivocally identified and isolated in both mice and humans (Spangrude *et al.*, 1988; Baum *et al.*, 1992). (A major technique used to achieve this is fluorescence-activated cell sorting (FACS), known as flow cytometry, a process by which different sub-populations of cells are identified and sorted away from a larger population, based on molecules (markers) on the cell surface that can be labeled with fluorescently-labeled antibodies. The sub-population of interest can then be characterized and further studied.) Realization that the human hematopoietic stem could self-renew led to the development of an entire field within medicine, hematopoietic stem cell transplantation. Patients who undergo this procedure develop and maintain a completely reconstituted hematopoietic system, comprised exclusively of the progeny of the donor's hematopoietic stem cells. The hematopoietic stem cells are rare; they constitute only 0.01–0.05% of the mouse bone marrow cells (Spangrude *et al.*, 1988; Baum *et al.*, 1992; Morrison *et al.*, 1995), and less than 1.1% of human bone

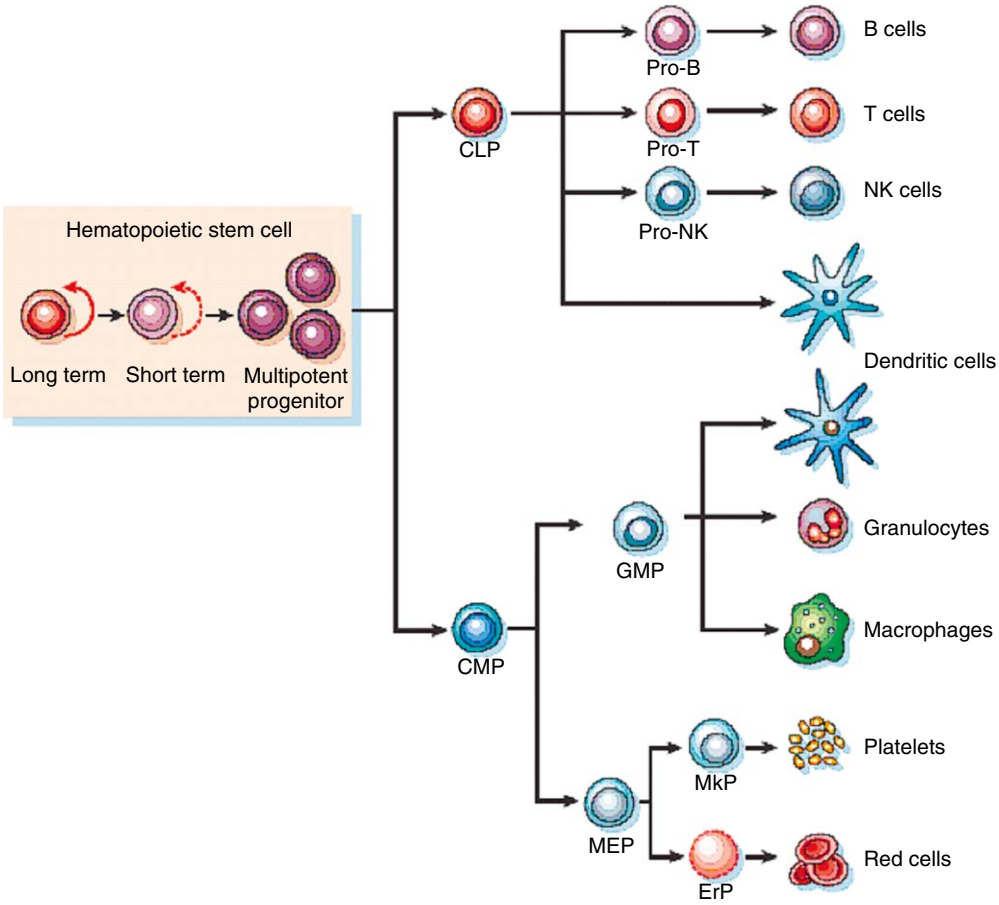


FIGURE 53-1 Self-renewal and differentiation in hematopoiesis. Long-term hematopoietic stem cells (HSCs) can self-renew for the lifetime of an animal (red arrows indicate self-renewal). Short-term HSCs have only limited self-renewal capacity. In contrast, multipotent progenitors do not have self-renewal capacity. However, in reference to differentiation, long-term self-renewing HSCs, short-term self-renewing HSCs, and multipotent progenitors all have the ability to give rise to all lineages of the blood system (shaded box). (CLPs: the precursors of all lymphoid cells; CMPs: the precursors of all myeloid cells; ErP: erythrocyte precursor; MEP: megakaryocyte erythrocyte precursor; GMP: granulocyte/macrophage progenitor; MkP: megakaryocyte precursor; NK: natural killer cell.) Modified from Reya *et al.* (2001). *Nature*, *414*, 105–111.

marrow cells (Baum *et al.*, 1992; Fruehauf and Seggewiss, 2003). By contrast, approximately 5% of gut epithelial cells are stem cells, demonstrating that tissue stem cells need not be as rare as blood stem cells in all tissues (Barker *et al.*, 2007).

Aside from the hematopoietic system, stem cells have now been prospectively identified in the adult tissues of both invertebrates and vertebrates (Clarke and Fuller, 2006; Morrison and Kimble, 2006). Relevant to cancer biology, mammalian stem cells have been identified in many of the tissues that commonly give rise to cancers, including the skin, the nervous system, the hematopoietic system, the breast, prostate, and the intestine (Spangrude *et al.*, 1988; Baum *et al.*, 1992; Stemple and Anderson, 1992; Kordon and Smith, 1998; Morrison *et al.*, 1999; Taylor *et al.*, 2000; Blanpain *et al.*, 2004; Stingl *et al.*, 2006; Barker *et al.*, 2007; Lawson *et al.*, 2007; Sangiorgi and Capecchi, 2008). Thus, self-renewal is an ancient and widespread process, and it is likely to involve evolutionarily conserved pathways.

NOT ALL CANCER CELLS ARE TUMORIGENIC: CLINICAL AND EXPERIMENTAL EVIDENCE

Under the CSC model, only a subset of cells with a tumor is able to self-renew and generate at least a part of a tumor's developmental heterogeneity: other malignant cells within the tumor should be non-tumorigenic. A number of independent observations, both in the clinic and in the laboratory, support this prediction. (Of note, these observations taken alone do not prove the CSC model, for the failure of a cancer cell to develop into a tumor could be due to an intrinsic inability of that cell to self-renew, or to the absence of necessary signals in the immediate microenvironment.)

The experiments of Southam and Brunschwig are among the most startling in cancer biology (Southam *et al.*, 1966). At that time, physicians had realized that malignant cells could be found circulating in the blood or lymphatics of cancer patients, but only a small percentage of these seemed to give rise to metastatic tumors. Southam

and Brunschwig sought to test this observation directly by harvesting tumor cells from patients with various disseminated or unresectable cancers, dissociating tumor fragments into single cell suspensions, and then subcutaneously autotransplanting varying numbers of cells to roughly determine how many tumor cells were needed to generate a new tumor. In spite of the deep ethical and methodological flaws, this study did suggest that, using their methods, only a subset of cells within a tumor is tumorigenic.

A similar observation has been made by clinicians providing palliative care to cancer patients with malignant ascites. In such patients who have implantation of a peritoneo-venous shunt—a procedure which can help alleviate abdominal distention and discomfort, but also has the unavoidable effect of shunting huge numbers of tumor cells into the systemic circulation—the development of disseminated cancer is an exceedingly rare event (Tarin *et al.*, 1984; Campioni *et al.*, 1986). A related clinical example is the recognition that viable breast cancer cells, the identity of which has been confirmed by immunocytochemical as well as genetic analysis, have been found in the bone marrow of a large percentage of breast cancer patients. Although in many patients the presence of these cells correlates with an increased probability of overt metastasis and a worse overall prognosis, the vast majority of these cells either remain dormant for years or are simply not tumorigenic (Braun *et al.*, 2005; Riethdorf *et al.*, 2008). Another supportive example is the observation that percutaneous biopsy of breast and liver tumors frequently leads to the disruption of the tumor and the deposition of thousands of malignant cells along the biopsy track, yet even with large bore needles, the rate of tumor seeding of the biopsy track is relatively low (Ryd *et al.*, 1983; Eriksson *et al.*, 1984; Smith, 1984; Diaz *et al.*, 1999; Silva *et al.*, 2008). Several possibilities could explain this discrepancy, including the idea that only a fraction of the tumor cells are tumorigenic.

Along these lines, in many different animal models of cancer—both with hematologic malignancies and solid tumors—a large number of tumor cells must be transplanted into a recipient animal order to generate a tumor (Bruce and Van Der Gaag, 1963; Hewitt *et al.*, 1976; Cho *et al.*, 2008). Similarly, only a minority of tumor cells is able to proliferate in vitro and generate colonies (Hamburger and Salmon, 1977a; Hamburger and Salmon, 1977b; Hill and Milas, 1989)

SOME CANCERS CAN DIFFERENTIATE INTO NONMALIGNANT CELLS

The CSC model implies that tumor heterogeneity is, at least in part, due to the execution of a developmental program in the progeny of cancer stem cells. As defined, cancer stem cells should be immature, multipotent, and able to give rise to both tumorigenic and non-tumorigenic cells. This has been shown in the case of mouse teratocarcinoma, a malignant germ cell tumor that can give rise to differentiated cell types of all three germ layers. When this tumor develops (it can occur spontaneously or be induced), it will metastasize and kill the host within a matter of weeks. Single cells, when injected subcutaneously into recipient mice, cause tumors to form. However, when single genetically-marked teratocarcinoma cells are isolated and injected into a normal mouse blastocyst, the progeny of the teratocarcinoma cells contribute functioning terminally-differentiated post-mitotic cells to multiple different tissues without inducing more tumors (Mintz and Illmensee, 1975; Illmensee and Mintz, 1976).

Importantly, among cancers, teratocarcinomas are not unique in their ability to execute a program of differentiation. Experiments with a transplantable squamous cell cancer in rats showed that tumor growth is driven mainly by immature, proliferating cells that subsequently develop into differentiated cells that, when transplanted, lose their tumorigenicity (Pierce and Wallace, 1971). Also, supportive data in humans comes from the genetic analysis of leukemias, in which genetic changes found in the leukemic clone can also be seen in mature, well-differentiated cells of various lineages. This suggests that the cancer is driven by immature hematopoietic cells that retain the capability of generating mature, noncancerous progeny (Fialkow, 1972; Fialkow *et al.*, 1977).

EVIDENCE SUPPORTING THE CANCER STEM CELL MODEL: PROSPECTIVE ISOLATION OF CANCER STEM CELLS

Prospective Isolation of Leukemia-initiating Cells

The demonstration that only some cells within many tumors are tumorigenic, and that malignant cells can elaborate developmental programs, provides strong evidence that supports the CSC model. But understanding the molecular controls that permit tumorigenicity requires the prospective isolation and purification of CSC so that they can be characterized and further studied.

Using the tools and strategies used to isolate normal stem hematopoietic stem cells (Spangrude *et al.*, 1988), this was first accomplished in acute myelogenous leukemia (AML) with the isolation of leukemia-initiating cells (L-IC) (Lapidot *et al.*, 1994; Bonnet and Dick, 1997). In AML, the peripheral blood and bone marrow are overpopulated with leukemic blasts, but these cells had demonstrated only a limited proliferative capacity *in vitro*. One possible explanation for this result was the existence of rare leukemia-initiating cells that drive the disease—cancer stem cells distinct from the leukemic blasts. (A potentially

confusing point in the literature is that the term "leukemia stem cell" does not necessarily imply an ontogenic relationship to normal hematopoietic stem cells.)

The general experimental approach, the xenograft assay (Figure 53-2), was to use cell surface markers to label primary human leukemia (AML) cells, perform flow cytometry to isolate distinct sub-populations, inject the different sub-populations into immunocompromised NOD–SCID (non-obese diabetic–severe combined immunodeficiency) recipient mice to show tumorigenicity of one sub-population, and to then repeat the process using the reconstituted leukemia to demonstrate that the tumorigenic sub-population was indeed capable of self-renewal. The L-ICs were found in the CD34+CD38− population; the other blasts were not capable of engraftment. Importantly, both the reconstituted and serially-transplanted leukemias that engrafted in the NOD–SCID mice preserved the surface marker heterogeneity of the parent malignancy; thus, this was not simply an assay of proliferation. Rather, it suggested a developmental program may be intrinsic to the L-ICs. And in fact, this was later shown in lineage tracing experiments with individual L-ICs (Hope *et al.*, 2004). Interestingly, CD34+CD38− is the same immunophenotype exhibited by normal hematopoietic stem cells, suggesting an ontogenic relationship between leukemia-initiating cells and normal hematopoietic stem cells. (Actually, more recent evidence suggests that the L-ICs from most patients phenotypically resemble early progenitor cells rather than normal HSCs, since (like progenitors) the L-ICs do not express CD90, i.e., they are CD34+CD38−CD90−, while normal HSCs do express CD90, i.e., they are CD34+CD38−CD90+ (Majeti *et al.*, 2007).) Regardless, because only the L-IC compartment remained small in both the parent leukemia and in the serial transplants into immunocompromised hosts, at least some constraints on self-renewal still remained intact in the leukemia.

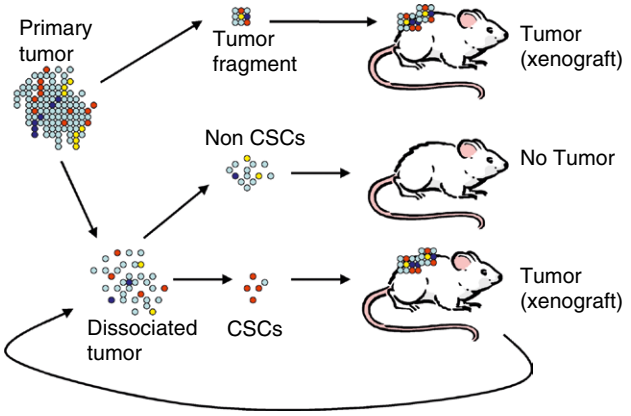


FIGURE 53-2 The xenograft assay (for solid tumors). A heterogeneous primary tumor is removed from a mouse or a human. A piece of the tumor can be directly implanted subcutaneously onto the back of a recipient mouse, and this will reliably lead to tumor growth. Alternatively, the tumor can be mechanically and enzymatically digested into a single-cell suspension, which can then be sorted by flow cytometry into different populations based on surface markers. One population may be enriched for cancer stem cells (CSCs, shown in red) and will give rise to a heterogeneous tumor in a recipient mouse, while the non-CSC population is non-tumorigenic. The xenografted tumor originating from cancer stem cells is then harvested, and the procedure is repeated to demonstrate that the CSCs have generated a heterogeneous tumor, which itself has CSCs and nonCSCs, and thus has the property of self-renewal. The xenograft assay is very similar for leukemias, where CSCs (otherwise known as leukemia initiating cells) are injected into the tail vein of the recipient mouse, rather than injected heterotopically.

Prospective Isolation of Solid Tumor Cancer Stem Cell

Subsequently, cancer stem cells (CSC) were prospectively isolated from solid tumors—first achieved in human breast cancer (Al-Hajj *et al.*, 2003). This was done by mechanically and enzymatically digesting primary tumors into single cell suspensions, and conducting xenograft assays on various sub-populations separated by flow cytometry (Figure 53-2). Such experiments revealed that only a percentage of tumor cells retained tumorigenic capacity. An empiric search for surface markers that would identify tumorigenic or nontumorigenic subpopulations showed that in eight out of nine patients' tumors, the tumorigenic fraction was in the CD44+CD24low/-Lineage-compartment. Cells outside of this compartment were non-tumorigenic. As few as 100 cells with the tumorigenic immunophenotype formed a tumor. Importantly, the xenograft tumors were heterogeneous, and themselves contained both tumorigenic and non-tumorigenic fractions, showing again that this was not merely a proliferation assay. The tumorigenic fraction could be used to passage the tumor serially, suggesting that the tumorigenic cells retained both self-renewal and the ability to execute a developmental program. Furthermore, because serially-passaged tumors continued to resemble their parent tumor, tumor heterogeneity was unlikely to be due exclusively to random mutations in different subclones of tumor cells (Figure 53-3).

Since then, using the xenograft assay, tumorigenic fractions of cells have been found in multiple different human solid tumors, including various nervous system tumors, colon cancer, hepatocellular carcinoma, pancreatic cancer, prostate cancer, and squamous cell cancer of the head and neck (Singh *et al.*, 2004; Collins *et al.*, 2005; Dalerba *et al.*, 2007; Li *et al.*, 2007; O'Brien *et al.*, 2007; Prince *et al.*, 2007; Ricci-Vitiani *et al.*, 2007). Different cell surface markers label the tumorigenic fraction in each system. For example, glioblastoma multiforme is a highly-aggressive human brain tumor containing cells that are both positive and negative for the surface marker CD133. In xenograft assays, the tumorigenic cells are contained within the fraction positive for CD133, comprising less than 30% of the tumor cells (Singh *et al.*, 2004). Similarly, in colon

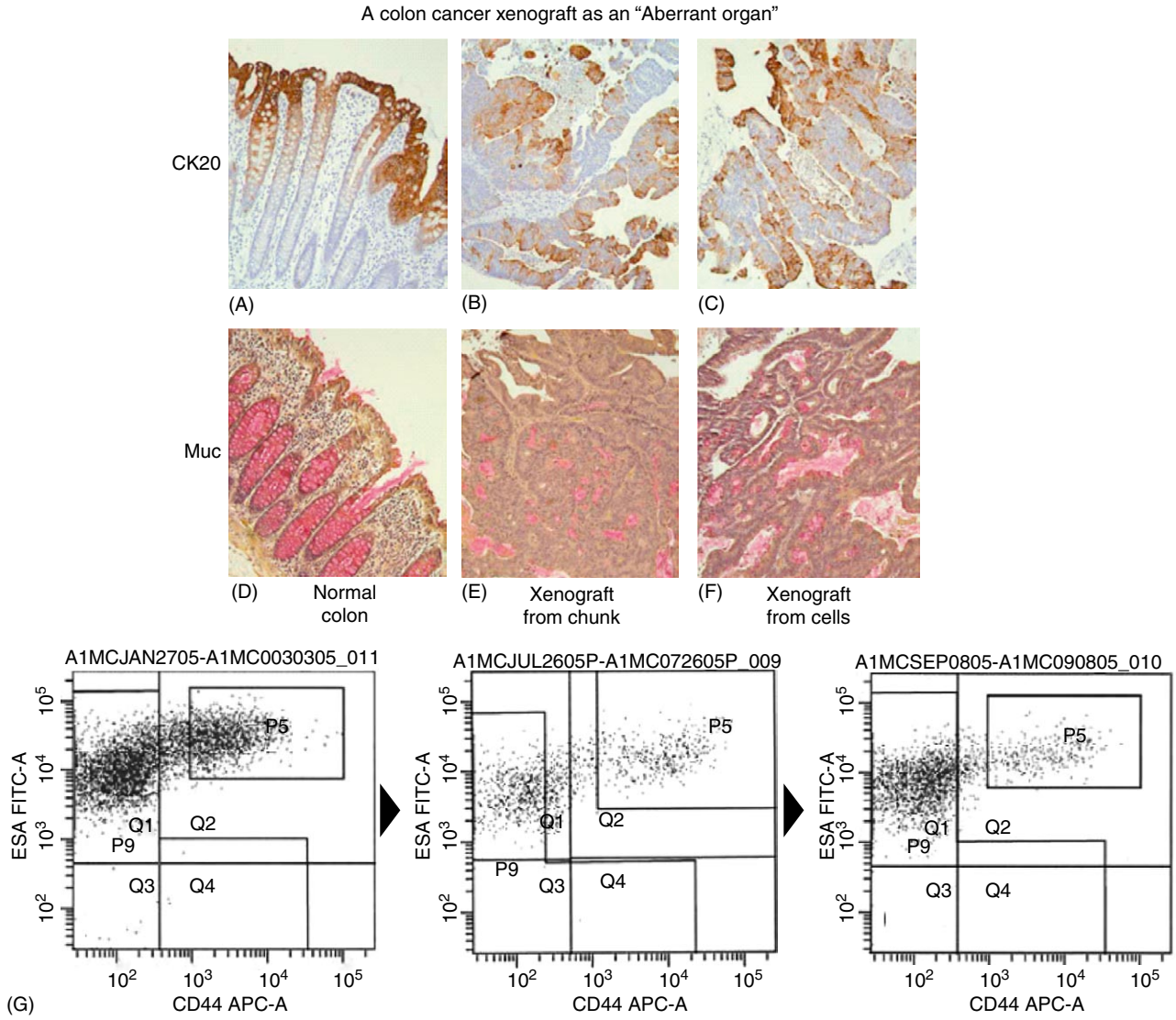


FIGURE 53-3 Shown are stainings of normal and colorectal carcinoma (CRC) samples with cytokeratin 20 (CK20) and neural epithelial mucins (Muc). (A, D) Normal epithelium stained with CK20 and Muc. (B, E) and (C, F) Xenografted human CRCs in NOD–SCID mice. The xenografts are differentiated adenocarcinomas forming gland-like structures reminiscent of normal colon. (B, E) Xenografts resulting from tumor fragments (undissociated pieces of tumor). (C, F) From sorted cancer stem cells. Resultant tumors from cancer stem cells show heterogeneity of cells as shown by staining and morphology. Bottom: (G) FACS plots from serially-passaged xenografts (the same tumor as shown in B, C, E, F), using ESA (y-axis) and CD44 (x-axis), two markers of colon cancer stem cells. Each FACS plot is from a different passage of the same parent tumor, and shows that the overall pattern remains constant. Modified from Dalerba *et al.* (2007). *Proc. Nat. Acad. Sci. USA*, *104*, 10158–10163.

cancer, different groups have found tumorigenic cells within the CD133+ sub-population, while others have observed them in the CD44+CD166+EpCAM+ population (Dalerba *et al.*, 2007; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007). In pancreatic cancers, the tumorigenic cells have the immunophenotype CD44+CD24+ESA+ (Li *et al.*, 2007).

It is important to consider that the surface markers merely serve as tools to mark the tumorigenic fraction. They may not actually play a role in self-renewal, tumorigenesis, or differentiation, and they may not mark the tumorigenic cells in all tumors. Furthermore, the markers used for cancer stem cells in most tumors are useful for enriching, but not purifying, the apparent stem cell compartment. Most likely not all of the cells isolated with the present markers are stem cells. Also, the other cellular compartments, although depleted for stem cells, may still contain some stem cells. Importantly, the present markers do not necessarily represent a specific state of differentiation, but rather were preferentially expressed by the stem cells in the tissue from which they were isolated, such as pleural effusions in the case of most breast cancers. Ultimately, cancer stem cells from different tissues may share certain molecular properties; for example, CD44 is

expressed in the cancer stem cells of most human epithelial cancers analyzed to date.

Clinical Relevance of Cancer Stem Cells

The tumorigenic cells isolated in the xenograft assay are clinically relevant. Gene expression profiling of the CD44+CD24low/− tumorigenic population in breast tumors has been used to generate an "invasiveness" gene signature (Liu *et al.*, 2007). Interestingly, this profile is associated with overall survival and metastasis-free survival, and predicts clinical outcomes in breast cancer independent of other established clinical and pathological characteristics. A related finding was made in the case of a mouse model of breast cancer, the MMTV-Wnt1 mouse. In spontaneous breast tumors from these mice, the tumorigenic cells exhibit a Thy1+CD24+CD49f+CD45− immunophenotype, and as few as 50 cells can generate a heterogeneous tumor in a syngeneic recipient mouse. Genes differentially expressed in tumorigenic and non-tumorigenic cells were used to generate an expression signature that predicted survival of human breast cancer patients (Cho *et al.*, 2008). Because these experiments dealt with mouse tumor cells being transplanted into syngeneic mouse recipients, they suggest that the identification of tumorigenic sub-populations of human breast cancer cells in xenograft assays are unlikely to be an artifact of transplanting human cells into mice. Furthermore, genes important in cancer stem cells in mouse models are likely to be relevant to human cancer biology.

Surface markers can be used to isolate a tumorigenic population of cells that is likely to be highly-enriched for cancer stem cells, but individual cancer stem cells have not yet been isolated. Undoubtedly, the tumorigenic cell populations are heterogeneous, and future studies will attempt to identify and characterize cancer stem cells more precisely.

ORIGINS OF CANCER STEM CELLS: THE PLASTICITY OF SELF-RENEWAL

Cancer Originating from Stem or Progenitor Cells

As described above, both normal tissue stem cells and cancer stem cells, by definition, are special cells that are able to self-renew and generate a heterogeneous tissue. The finding that leukemic stem cells in AML share the same immunophenotype with normal hematopoietic stem cells, CD34+CD38−, might suggest that cancers result from the oncogenic transformation of normal stem cells. Similarly, observations about the time from carcinogenic exposure to the development of cancer also seem to support the notion that cancers derive from normal tissue stem cells. For example, if a person is exposed to a carcinogen for a finite period of time, many years may elapse between the end of the exposure and the eventual development of cancer. This has been shown for solar ultraviolet light exposure and the later development of malignant melanoma (Brash and Ponten, 1998), as well as cigarette smoke exposure and the later development of lung cancer (Thun *et al.*, 2002). Traditionally, the only cells in a tissue thought to persist long enough to accumulate several oncogenic mutations, and exhibit such a delayed effect, were the tissue stem cells. Because of their rapid turnover, any progenitor cells, i.e., non-stem cells, that acquire mutations would be expected to die off before enough mutations could accumulate to transform them—unless, of course, the very mutations that cause cancer also hinder the mechanisms that normally limit proliferation in progenitor cells and drive their maturation and senescence. This is born out in a mouse model of human intestinal tumors. The initiating mutation of most human colon cancers is loss of the APC tumor suppressor gene (Kinzler and Vogelstein, 1996). Elegant experiments by Clevers and colleagues show that loss of APC in intestinal progenitor cells results in small, benign lesions, whereas loss of APC in colon stem cells gives rise to malignant lesions (Clevers, unpublished data). Although the initiating lesion is likely to arise in the normal stem cells, other recent studies suggest that cancer stem cells are not necessarily descended from normal tissue stem cells. They can also develop from progenitor cells, i.e., non-self-renewing cells that acquire the capacity for self-renewal (Figure 53-4).

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by the uncontrolled proliferation of granulocytes. It accounts for 15–20% of leukemia in adults. The disease has a relatively indolent course until it enters an accelerated phase, and then a "blast

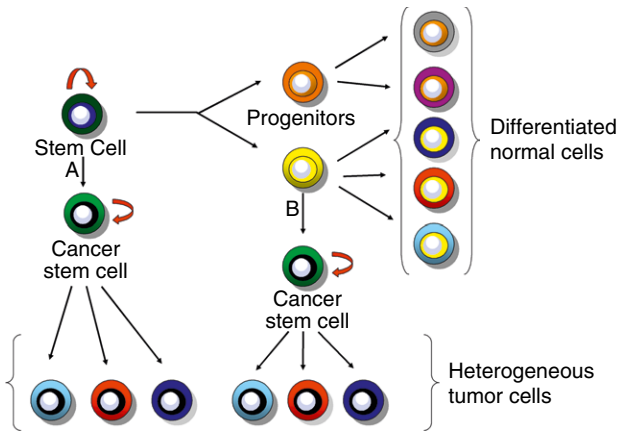


FIGURE 53-4 Origins of cancer stem cells. Cancer stem cells may originate from transformed stem cells (A), or progenitor cells (B). If they originate from progenitor cells, they gain self-renewal properties. In normal tissues, progenitor cells can acquire self-renewal through mutation of several genes.

crisis" phase which resembles acute leukemia. The cell of origin in CML was originally thought to be a hematopoietic stem cell (HSC) since the Philadelphia chromosome, t(9;22), the characteristic chromosomal translocation of CML which leads to the production of the oncogenic fusion protein Bcr-Abl, can be detected in HSCs in CML patients. However, solid evidence for malignant HSCs in CML has been lacking, and a mouse model of CML has shown that blast crisis can develop even when the Bcr-Abl fusion protein is driven in progenitor cells rather than HSCs (Jaiswal *et al.*, 2003). Recently, careful surface marker studies of leukemic cells in blast crisis CML have shown that the self-renewing leukemic cells exhibit a cell surface profile shared by myeloid progenitor cells rather than HSCs (Jamieson *et al.*, 2004).

In AML, further characterization of the immunophenotype of leukemia-initiating cells also supports the hypothesis that L-ICs may originate from progenitors rather than stem cells. Although both HSCs and L-ICs are CD34+CD38−, unlike normal HSCs, L-ICs do not express CD90, and thus more closely resemble progenitors rather than stem cells (Majeti *et al.*, 2007). This principle has also been supported by recent studies in solid tumors. For example, in a mouse model of medulloblastoma driven by Hedgehog signaling, the acquisition of a progenitor cell fate is an essential step in tumorigenesis. This suggests that CNS progenitor cells (i.e., cerebellar granule neuron precursors) rather than neural stem cells (i.e., multipotent ventricular zone cells) are the likely cell of origin in this CNS malignancy (Schuller *et al.*, 2008; Yang *et al.*, 2008).

Mechanisms for Acquiring Self-renewal

If a cancer, which by definition must contain self-renewing cells, can arise from progenitor cells, then self-renewal must be an attribute that non-self-renewing cells can acquire. How can this occur? Recent studies have shown that Bmi1 and its downstream targets may be involved. Bmi1 is a Polycomb group gene that normally functions by epigenetically repressing genes that positively regulate senescence, differentiation, and cell death. When HSCs are mutant for Bmi1, they quickly exhaust after several weeks, rather than continually self-renewing. Thus, Bmi1 is required to maintain self-renewal in HSCs (Lessard and Sauvageau, 2003; Park *et al.*, 2003) (Bmi1 may be a more general self-renewal factor: it is required for maintenance of self-renewal in neural stem cells, marks intestinal stem cells, and is expressed in many tissues that continuously regenerate (Molofsky *et al.*, 2003; Hosen *et al.*, 2007; Sangiorgi and Capecchi, 2008).) Remarkably, when three major downstream targets of Bmi1 are mutated (p53, p16-Ink4a, and p19-ARF), progenitor cells gain the ability to self-renew (Akala *et al.*, 2008). This suggests a mechanism by which progenitor cells might adopt a self-renewing fate to become cancer stem cells (Figure 53-4). Interestingly, mutations of p53, p16-Ink4a, and p19-ARF are commonly found in cancers, hinting that progenitor cells may be the origin of cancer stem cells more broadly.

The loss of function of genes that positively regulate senescence, differentiation, and cell death may not be the only mechanism to enable non stem-cells to obtain a self-renewing phenotype. Several groups have recently demonstrated that the expression of a small number of defined transcription factors (for example, Oct3/4, Sox2, Klf4, and c-Myc) can enable terminally-differentiated somatic cells (fibroblasts) to become pluripotent, adopt an embryonic stem cell-like fate, and thus obtain the property of self-renewal (Takahashi and Yamanaka, 2006; Okita *et al.*, 2007; Wernig *et al.*, 2007). Remarkably, this has been shown with both mouse and human somatic cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007). More recently, this has been achieved in human fibroblasts with only two non-oncogenic transcription factors, Oct3/4 and Sox2, in the presence of a chemical inhibitor of histone deacetylase, valproic acid (Huangfu *et al.*, 2008).

Even though terminally-differentiated cells such as fibroblasts possess the capacity to be reprogrammed and adopt a self-renewing fate, as of now there is no evidence that cancer stem cells actually utilize this mechanism to acquire self-renewal. Indeed they may not, since the *in vivo* reprogramming of somatic cells would likely lead to germ cell tumors (Thomson *et al.*, 1998; Takahashi and Yamanaka, 2006; Maherali *et al.*, 2007; Wernig *et al.*, 2007), but such tumors generally do not arise in differentiated tissues such as breast, colon, prostate, lung, etc. However, it remains possible that the partial reprogramming of a differentiated cell could activate the self-renewal machinery. Several genes that are required for maintaining pluripotency and driving rapid proliferation in embryonic stem cells are frequently up-regulated in cancer, including c-Myc, Beta-catenin, Stat3, Klf4, and E-ras (Niwa *et al.*, 1998; Matsuda *et al.*, 1999; Kielman *et al.*, 2002; Takahashi *et al.*, 2003; Sato *et al.*, 2004; Cartwright *et al.*, 2005; Li *et al.*, 2005).

SIGNALING PATHWAYS IN CANCER STEM CELLS

Evolutionarily divergent metazoans—including the worm *C. elegans*, the fruitfly *Drosophila melanogaster*, the mouse *Mus musculus*, and the human *Homo sapien*—possess stem cells that maintain various adult tissues. This observation has led to the hypothesis that certain highly-conserved genes may contain an evolutionarily-conserved self-renewal machinery. Such reasoning has in turn led stem cell biologists to hypothesize that normal and cancer stem cells share at least some molecular mechanisms of self-renewal. This makes sense, since the maintenance of stem cells—whether

normal or cancerous—involves the repression of pathways for differentiation, senescence, and apoptosis (which themselves are highly conserved). Thus, positive regulators or effectors of these pathways should be quiescent in stem cells, while repressors of such pathways should be active or overexpressed. This has been demonstrated in HSCs, where the molecular mechanisms underlying self-renewal have been most extensively characterized (Akala and Clarke, 2006).

Several observations provide indirect support for the idea that mechanisms of self-renewal are shared by CSCs and normal tissue stem cells. For example, in cases where the immunophenotype of CSCs resembles that of the normal tissue stem cells, a parsimonious model of tumorigenesis might suggest that, as the normal stem cell becomes cancerous, the self-renewal machinery simply continues to function. This idea is supported by the observation that both normal hematopoietic stem cells and leukemia stem cells require the same gene, Bmi1, for self-renewal (Lessard and Sauvageau, 2003; Park *et al.*, 2003). Similarly, Wnt signaling has been implicated in self-renewal, in both normal hematopoietic stem cells and leukemia initiating cells (Reya *et al.*, 2003; Jamieson *et al.*, 2004). However, even if cancer stem cells are not always descended directly from normal tissue stem cells (as described above), they may still share mechanisms of self-renewal. Many genes involved in the pathways that regulate self-renewal in normal stem cells (such as Hedgehog, Wnt/Beta-catenin, Notch, and Bmi1), are often implicated in neoplasia.

For example, in the intestine, Wnt signaling has been shown to be an important pathway regulating tissue architecture, possibly (but not definitely) due to effects on stem cells (Gregorieff and Clevers, 2005; Reya and Clevers, 2005). In the intestinal epithelium, reducing Wnt signaling by mutating β-catenin, mutating Tcf-4, or overexpressing Dickkopf-1 all lead to severe tissue disruption and loss of proliferating epithelial cells, as one would predict if Wnt signaling were required for intestinal stem cell renewal (Korinek *et al.*, 1998; Pinto *et al.*, 2003; Ireland *et al.*, 2004; Kuhnert *et al.*, 2004). Recently, the intestinal stem cells were unambiguously identified based on their expression of Lgr5, a Wnt target gene (Barker *et al.*, 2007). As in hematopoietic stem cells, Wnt signaling is likely playing an important role in normal intestinal stem cells, perhaps by regulating self-renewal—although definitive evidence that the pathway regulates self-renewal in the intestine is still lacking. In colon cancer tumorigenesis, Wnt signaling has long been recognized as an important pathway, in large part because patients with Familial Adenomatous Polyposis invariably develop colon adenocarcinoma. These patients are heterozygous for loss of function mutations in APC, a key molecule in canonical Wnt signaling, and their colon cancers exhibit severe loss of APC (by loss of the normal allele) and constitutive Wnt signaling (as indicated by nuclear β-catenin staining) (Gregorieff and Clevers, 2005). Thus, it seems likely that Wnt signaling regulates self-renewal in both normal intestinal stem cells and colon CSCs.

Since cancer stem cells were only recently isolated, very little is definitively known about the molecular pathways involved in their regulation. Many pathways shown to have a role in self-renewal in normal stem cells may or may not have a role in cancer stem cells, and genes involved in self-renewal of normal stem cells may serve a different function in cancer stem cells.

For example, distinct from its role in embryonic patterning, Hedgehog signaling has been implicated in the regulation of self-renewal in adult tissues (Taipale and Beachy, 2001). Sonic Hedgehog (Shh) triggers increased self-renewal and proliferation in HSCs (Bhardwaj *et al.*, 2001), and also in neural stem cells, perhaps via activation of Bmi1 (Leung *et al.*, 2004). (Interestingly, Hedgehog signaling does not appear to be required for self-renewal in normal HSCs, since HSCs mutant for Smoothened, an important cofactor in Hedgehog signaling, are not defective in long-term hematopoietic reconstitution (Dierks *et al.*, 2008).) Hedgehog signaling has also been implicated in the tumorigenesis of several cancers, including basal cell carcinoma, medulloblastoma, and others (Taipale and Beachy, 2001; Liu *et al.*, 2006). In breast cancer stem cells and CML stem cells, Hedgehog signaling is active and involved in regulating proliferation and self-renewal (Liu *et al.*, 2006; Dierks *et al.*, 2008). But cancer stem cells may utilize Hedgehog signaling for other processes besides self-renewal. In pancreatic cancer stem cells, experiments with small molecule inhibitors of Hedgehog signaling suggest that Hedgehog signaling may have a more predominant effect on invasion and metastasis (Feldmann *et al.*, 2007). Many pathways have been implicated in the regulation of self-renewal in normal tissues, including Wnt, Notch, Hedgehog, BMP, Jak-Stat, AKT/PI3K, Polycomb group proteins, and others (Akala and Clarke, 2006; Lobo *et al.*, 2007; Visvader and Lindeman, 2008). Most of these have also been implicated in tumorigenesis. However, because cancer stem cells are not the same as normal tissue stem cells, they may utilize various pathways differently. In the end, it is likely that some mechanisms of self-renewal will be shared, and others will not. Regardless of whether normal and cancer stem cells share mechanisms of self-renewal, the identification of molecular pathways involved in the maintenance of cancer stem cells may present therapeutic opportunities.

CLINICAL IMPLICATIONS OF CANCER STEM CELLS

The cancer stem cell model has several important clinical implications, some of which may be profound. It might not only contribute a novel pathophysiological mechanism to explain tumors' resistance to treatment, but it might also

improve clinicians' ability to more effectively diagnose, prognose, and treat cancer.

Since the cancer stem cell model suggests that CSCs are responsible for the sustained and uncontrolled growth of malignancies, treatment failures in the clinic may be due partly to the resistance of cancer stem cells to therapy. If cancer stem cells are intrinsically more resistant to treatment (such as chemotherapy or radiotherapy) than other cells in the tumor, then treatment should increase the percentage of cancer stem cells relative to pretreatment levels. Conversely, if all cells are similarly sensitive to treatment, then the relative percentages of tumorigenic and non-tumorigenic cells should not change as a tumor shrinks.

In support of this prediction, a recent study examining early passage colon cancer xenografts showed that the CD44+ESA+ cells, the population containing the colon cancer stem cells, were significantly enriched after treatment with cyclophosphamide, a chemotherapeutic agent. Tumors that had been exposed to cyclophosphamide had an expanded CD44+ESA+ compartment, and also more tumorigenic cells relative to controls, as shown in a serial dilution study of tumorigenicity. Importantly, even after treatment, equal numbers of CSCs from the treatment and control arms were equally tumorigenic (Dylla *et al.*, 2008). Similarly, in human patients with locally advanced breast cancer, the frequency of CSCs as shown by immunophenotype, as well as the ability to form mammospheres *in vitro* (an assay that correlates with stem cell activity), significantly increased shortly after treatment (Li *et al.*, 2008).

The often-observed initial shrinkage of a tumor soon after treatment might reflect the relative sensitivity of the non-tumorigenic cells—those that comprise the bulk of the tumor—and may not be as important as is currently thought. Thus, the most important clinical variable for assessing treatment response and prognosis may not be absolute tumor size, but rather the absolute number of CSCs remaining after treatment. Similarly, tumors with high proportions of cancer stem cells might be more aggressive and difficult to treat. This is not to say that one should not attempt to eliminate most of the cancer cells in a tumor. The fact that certain mutations can enable normal progenitor cells to self-renew (Akala *et al.*, 2008) suggests that mutations could reactivate self-renewal programs in residual non-tumorigenic cancer cells in some patients. Nonetheless, elimination of the cancer stem cell population in a tumor could lead to dramatically better clinical outcomes.

Aside from their intrinsic resistance to treatment, CSCs are also likely to possess mechanisms for acquiring resistance to treatment, such as genetic instability. The various genetic changes known to occur in tumors—including point mutations, chromosomal rearrangements, aneuploidy, and gene amplifications—may occur within CSCs (Lengauer *et al.*, 1998). In fact, a study on tumorigenic colon cancer cells suggests that they can develop karyotypic changes (Odoux et al., 2008), but whether this occurs *in vivo* and leads to treatment resistance remains unproven.

FUTURE DIRECTIONS

Although the cancer stem cell model has existed for well over 50 years, during the past several years the techniques of modern stem cell biology have facilitated rapid progress in the field. Among the major goals is to further enrich tumorigenic cancer stem cells to a higher level of purity—ideally to a single-cell level—in order to demonstrate formally that a single cell can generate a heterogeneous cancer and self-renew. One current approach to this problem is to discover new markers; however, this is intrinsically difficult because of the time- and labor-intensive nature of the current assays, as well as the lack of simple forward genetic approaches. Finding new markers is empiric and difficult. Ultimately, achieving this goal may depend on the development of entirely new approaches, assays, and techniques—most likely drawing on knowledge from normal stem cell biology and working at the single-cell level.

The purification of CSCs would dramatically change the field. First, cancer stem cell biologists would simply be better able to characterize the cells—to understand more clearly what makes them unique. What genetic pathways do they utilize, and how do they use them? What is their origin? What do they need to maintain self-renewal? How do they go on to generate a developmental hierarchy? Which of the many genes and mutations previously described in cancer are important in the cancer stem cells? How often do they divide, and what regulates their division? Do they divide symmetrically, or asymmetrically, or both—and what regulates the choice? Where in the tumors can they be found, and what do they look like? Can they be imaged in live tumors? Do they migrate, and if so how? How do they interact with their niche? How do they contribute to metastasis?

Ultimately, the goal is to apply these insights to the clinic—to develop better diagnostic tests and more targeted and effective anti-cancer therapies. Developments in the field should lead to a deeper understanding of the mechanisms of resistance to chemotherapy and radiotherapy. And ideally, patients will have more opportunities to experience curative, rather than palliative, therapies.

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Chapter 54

Neural Stem Cells for Central Nervous System Repair

Rodolfo Gonzalez, Jean Pyo Lee, and Evan Y. Snyder

INTRODUCTION

Despite the presence of endogenous neural stem cells (NSCs) in the mammalian brain, it is recognized that intrinsic "self-repair" activity for the most devastating of injuries is inadequate or ineffective. This poor regenerative ability, particularly in the adult central nervous system (CNS), may be because of the limited number and restricted location of native NSCs, and/or limitations imposed by the surrounding microenvironment, which may not be supportive or instructive for neuronal differentiation. NSCs expanded *ex vivo* in culture, and then implanted into regions needing repair, may overcome those limitations related simply to inadequate numbers of NSCs near the defective region. Whether the environment may also inhibit exogenous NSCs from surviving or differentiating toward replacement cells is a possibility. However, several transplantation experiments have suggested that neurogenic cues are transiently elaborated during degenerative processes (perhaps recapitulating developmental cues), and that exogenous NSCs are able to sense, home in on, and respond appropriately to those. In other words, NSCs appear to respond *in vivo* to neurogenic signals, not only when they occur appropriately during development, but even when induced at later stages by certain neurodegenerative processes.

In this chapter, we review some of the work that has been performed in animal models of CNS diseases where transplanted NSCs have mediated a therapeutic effect. These disorders include rodent models of genetic and acquired (e.g., traumatic and ischemic) neurodegeneration, inheritable metabolic disorders, age-related degeneration, and neoplasms. These conditions often have widespread neural cell loss, dysfunction, or both. The disseminated nature of the pathology in these diseases is not readily treated by conventional transplantation approaches, in which a solid tissue graft or a limited number of non-motile cells are delivered to a restricted area. Similarly, most gene therapy approaches tend to fall short because of their limited "sphere of influence" following injection of the vector into the CNS parenchyma. The use of bone marrow transplantation, even with hematopoietic stem cells, is typically inadequate, because the cells do not efficiently broach the blood–brain barrier. Cells from non-neural organs, if they transdifferentiate into neural cells at all (a controversial prospect), do so far too inefficiently to be therapeutically reliable for cell replacement in these conditions.

NSCs, on the other hand, circumvent many of these obstacles. Because they differentiate robustly into neural cells, integrate seamlessly into neural parenchyma as multiple neural cell types (both neuronal and glial), respond to normal developmental and regeneration cues, and migrate (even long distances) to multiple, disseminated areas of neuropathology, NSCs appear to be ideally suited for the molecular and cellular therapies required by extensive, diffuse (even "global") degenerative processes. Examples of such widespread neurodegenerative conditions include myelin disorders, storage diseases, motor neuron degeneration, dementia conditions such as Alzheimer's disease, and ischemic and traumatic pathologies, such as stroke. Some diseases appear to be restricted in their involvement—Parkinson's disease is localized to the mesostriatum, Huntington's to the caudate, spinal cord contusion to a few spinal segments, and cerebellar degeneration to the hindbrain. However, even these disorders require cell replacement to be distributed evenly over relatively large areas, and that multiple neural cell types be replaced in a specific region, or both. Again, these needs are best accomplished by a migratory, responsive, multipotent neural progenitor, even if transplantation is directed toward a more circumscribed CNS region.

THERAPEUTIC POTENTIAL OF NEURAL STEM CELLS

The clinical potential of the NSC is rooted in its inherent biologic properties. The ability of NSCs and their

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progeny to develop into integral cytoarchitectural components of many regions throughout the host brain as neurons, oligodendrocytes, astrocytes, or even immature neural progenitors, makes them capable of replacing a range of missing or dysfunctional neural cells. Although the field of neural repair has tended to place emphasis on the replacement of missing neurons in acute and chronic neurodegenerative diseases, or oligodendrocytes in demyelinating disorders, it is becoming increasingly evident that simultaneously replacing a diseased cell's "neighboring cells"—typically astrocytes—may be of equal importance, because of the indispensable trophic, guidance, and detoxification role such "chaperone" cells may play. Fortuitously, part of the biological repertoire of an NSC is to generate the variety of cells that constitute the "fabric" of a given neural region. NSCs in particular differentiation states will spontaneously produce a variety of neurotrophic factors that may serve trophic, protective, or both functions—for example, glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3). NSCs also inherently express most of the "housekeeping" enzymes and factors necessary for any cell to maintain normal metabolism.

NSCs may also be readily engineered *ex vivo* to express a variety of molecules that either are not produced by NSCs, or are not produced in therapeutically adequate quantities. The NSCs are amenable to various types of viral vector transduction, as well as to other gene transfer strategies, such as lipofection, electroporation, and calcium-phosphate precipitation. Following transplantation, such engrafted NSCs may then be used as cellular vectors for the in vivo expression of exogenous genes of developmental and/or therapeutic relevance. Such gene products can be delivered either to circumscribed regions or, if necessary, to more widely-disseminated areas throughout the host CNS. Because they display significant migratory capacity, as well as an ability to integrate widely throughout the brain when implanted into germinal zones, NSCs may help to reconstitute enzyme and cellular deficiencies in a global fashion (Figure 54-1).

The ability of true NSCs to adjust to their regions of engraftment probably obviates the need for abstracting stem cells from specific CNS regions. Furthermore, NSCs appear to possess a tropism for degenerating CNS regions (Figure 54-2). NSCs may be attracted—across long distances—to regions of neurodegeneration in brains of all ages, including old age. Under some circumstances, an environment in which a particular type of neural cell has degenerated creates a milieu that directs the differentiation of an NSC toward maintaining homeostasis, including replenishment of a specific deficient neural cell type. Through mechanisms yet to be determined, it appears that some neurodegenerative processes (e.g., those associated with apoptosis) elaborate neurogenic signals that recapitulate developmental cues to which NSCs can respond (Figure 54-3).

It would seem that NSC-mediated cell replacement would only be feasible in "cell autonomous" disease

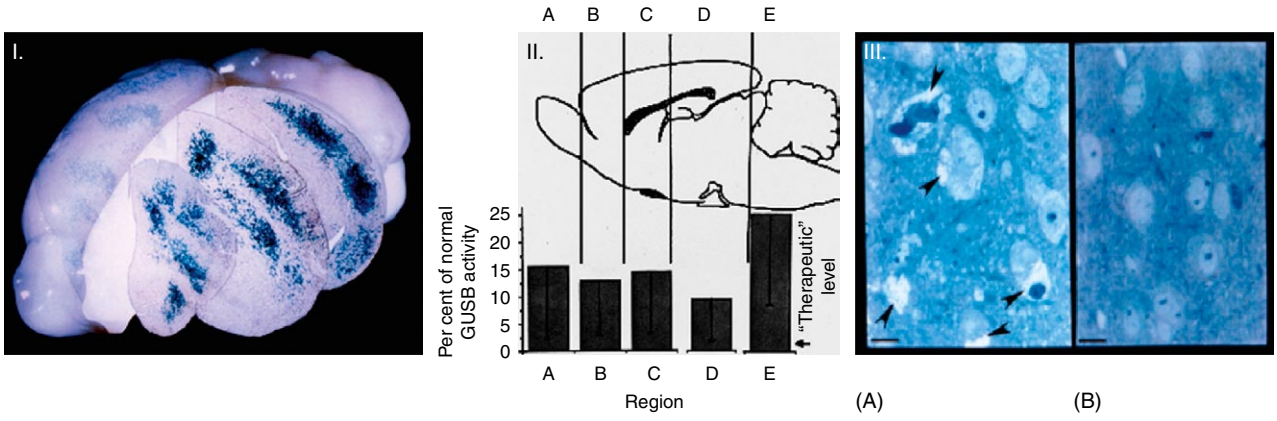


FIGURE 54-1 Widespread engraftment of NSCs expressing GUSB throughout the brain of the MPS VII mouse. (A) Brain of a mature MPS VII mouse after receiving a neonatal intraventricular transplant of murine NSCs expressing GUSB. Donor NSC-derived cells, identified by their X-gal histochemical reaction for expression of the *LacZ* marker gene, have engrafted throughout the recipient mutant brain. Coronal sections—placed at their appropriate level by computer—show these cells to span the rostral–caudal expanse of the brain. (B) Distribution of GUSB enzymatic activity throughout brains of MPS VII NSC transplant recipients. Serial sections were collected from throughout the brains of transplant recipients and assayed for GUSB activity. Sections were pooled to reflect the activity within the demarcated regions. The regions were defined by anatomical landmarks in the anterior-to-posterior plane to permit comparison among animals. The mean levels of GUSB activity for each region ($n = 17$) are presented as the percentage of average normal levels for each region. Untreated MPS VII mice show no GUSB activity, biochemically or histochemically. Enzyme activity of 2% of normal is corrective, based on data from liver and spleen. (C) Decreased lysosomal storage in a treated MPS VII mouse brain at eight months. (1) Extensive vacuolation representing distended lysosomes (arrowheads) in both neurons and glia in the neocortex of an 8-month-old, untransplanted control MPS VII mouse. (2) Decrease in lysosomal storage in the cortex of an MPS VII mouse treated at birth from a region analogous to the untreated control section in panel 1. The other regions of this animal's brain showed a similar decrease in storage compared with untreated, age-matched mutants in regions where GUSB was expressed. Scale bars = 21 μm.

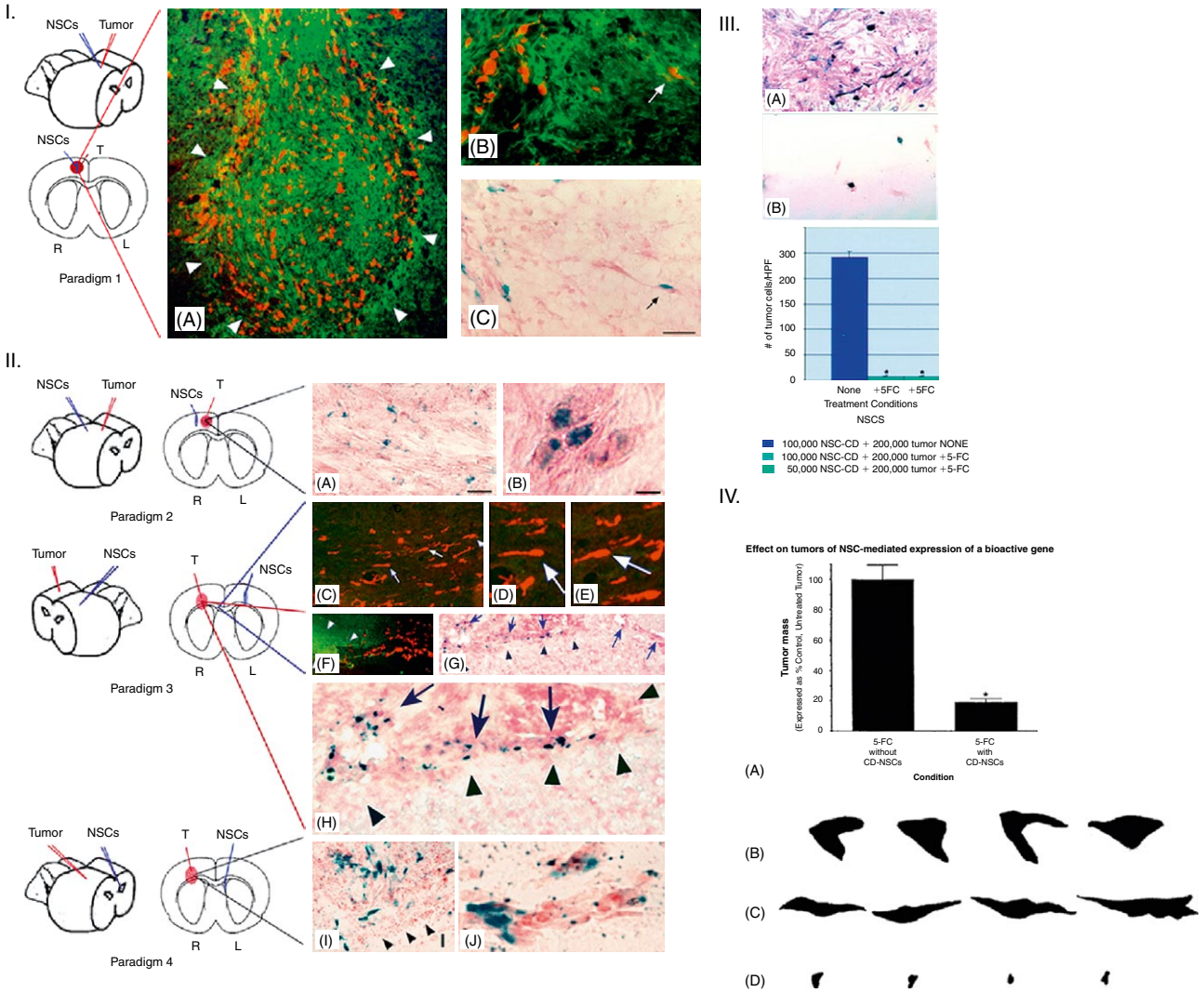


FIGURE 54-2 Neural stem cells display extensive tropism for pathology in the adult brain and can express bioactive genes within such pathological situations: evidence from intracranial gliomas. (A) NSCs migrate extensively throughout a brain tumor mass *in vivo* and "trail" advancing tumor cells. Paradigm (1), in which NSCs are implanted directly into an established experimental intracranial glioblastoma, is illustrated schematically. (1) The virulent and aggressively-invasive CNS-1 glioblastoma cell line, used to create the tumor, has been labeled *ex vivo* by transduction with GFP cDNA. The NSCs stably express *lacZ*, and produce βgal. This panel, processed for double immunofluorescence using an anti-βgal antibody (NSCs) and an anti-GFP antibody (glioblastoma cells), shows a section of brain (under low power) from an adult nude mouse sacrificed 10 days after NSC injection into the glioblastoma; arrowheads mark where the tumor mass interfaces with normal tissue. Donor βgal+ NSCs can be seen extensively distributed throughout the mass, interspersed among the tumor cells. This degree of interspersion by NSCs after injection occurs within 48 hours. Interestingly, although NSCs have extensively migrated and distributed themselves within the mass, they largely stop at the junction between tumor and normal tissue, except where a tumor cell is infiltrating normal tissue; then, NSCs appear to follow the invading tumor cell into surrounding tissue. (1 and 2) Detail of the trailing of individual glioblastoma cells migrating from the main tumor bed. (2) High power view, under fluorescence microscopy, of single migrating infiltrating GFP+ tumor cells in apposition to βgal+ NSCs (white arrow). (3) Costaining with Xgal (the arrow points to the *lacZ*-expressing NSCs) and with neutral red (for the elongated glioblastoma cells). The NSC is in direct juxtaposition to a single migrating, invading the neutral red+, spindle-shaped tumor cell (arrow), with the NSC "piggy-backing" the glioma cell. Scale bar = 60 μm. (B) NSCs implanted at various intracranial sites far from main tumor bed migrate through normal adult tissue toward glioblastoma cells. (1 and 2) Same hemisphere: A section through the tumor from an adult nude mouse six days following NSC implantation caudal to tumor. Panel 1 shows a tumor populated as pictured under low power in Figure 54-1A. Note Xgal+ NSCs interspersed among neutral red+ tumor cells. (2) High-power view of NSCs in juxtaposition to islands of tumor cells. (3–8) Contralateral hemisphere. (2–5) Views through the corpus callosum (c), where βgal+ immunopositive NSCs (arrows) are migrating from their site of implantation on one side of the brain toward tumor on the other. Two NSCs, indicated by arrows in panel 3, are viewed at higher magnification in panels 4 and 5 to show the classic elongated morphology and leading process of a migrating neural progenitor oriented toward its target. (6) βgal+ NSCs are "homing in" on the GFP+ tumor, having migrated from the other hemisphere. In panel 7, and magnified further in panel 8, the Xgal+ NSCs (arrows) have entered the neutral red+ tumor (arrowheads) from the opposite hemisphere. (9 and 10) Intraventricular: A section through the brain tumor of an adult nude mouse six days after NSC injection into the contralateral cerebral ventricle. (9) Xgal+ NSCs are distributed within the neutral red+ main tumor bed (edge delineated by arrowheads). (10) At higher power, the NSCs are in juxtaposition to migrating islands of glioblastoma cells. Fibroblast control cells never migrated from their injection site in any paradigm. All Xgal positivity was corroborated by anti-βgal immunoreactivity. Scale bars = 20 μm (1; also applies to 3) 8 μm (2), 14 μm (4 and 5), 30 μm (6 and 7), 15 μm (8), 20 μm (9), and 15 μm (10). (C) Bioactive transgene (cytosine

FIGURE 54-2 (*Continued*)
deaminase, CD) remains functional (as assayed by *in vitro* oncolysis) when expressed within NSCs. CNS-1 glioblastoma cells were co-cultured with murine CD-NSCs. (1) Co-cultures unexposed to 5-FC grew healthily and confluent, (2) whereas plates exposed to 5-FC showed dramatic loss of tumor cells represented quantitatively by the histograms (*= $p < 0.001$). The oncolytic effect was identical whether 1×10^5 CD-NSCs or half that number were co-cultured with a constant number of tumor cells. (Subconfluent NSCs were still mitotic at the time of 5-FC exposure, and thus subject to self-elimination by the generated 5-FU and its toxic metabolites.) (D) Expression of *CD* delivered by NSCs *in vivo* as assayed by reduction in tumor mass. The size of an intracranial glioblastoma populated with CD-NSCs in an adult nude mouse treated with 5-FC was compared with that of tumor treated with 5-FC, but lacking CD-NSCs. These data, standardized against and expressed as a percentage of a control tumor populated with CD-NSCs receiving no treatment, are in the histograms in panel 1. These measurements were derived from measuring the surface area of tumors; camera lucidas of them are in panels 2–4. Note the large areas of panel 2 a control non-5-FC-treated, tumor-containing CD-NSCs and in panel 3 a control 5-FC-treated, tumor-lacking CD-NSCs as compared to panel 4 the dramatically smaller tumor areas of the 5-FC-treated animal, which also received CD-NSCs (~80% reduction, * = $p < 0.001$), suggesting both activity and specificity of the transgene. (3) The lack of effect of 5-FC on tumor mass when no CD-bearing NSCs were within the tumor was identical to panel 4 the effect of CD-NSCs in the tumor without the gene being employed.

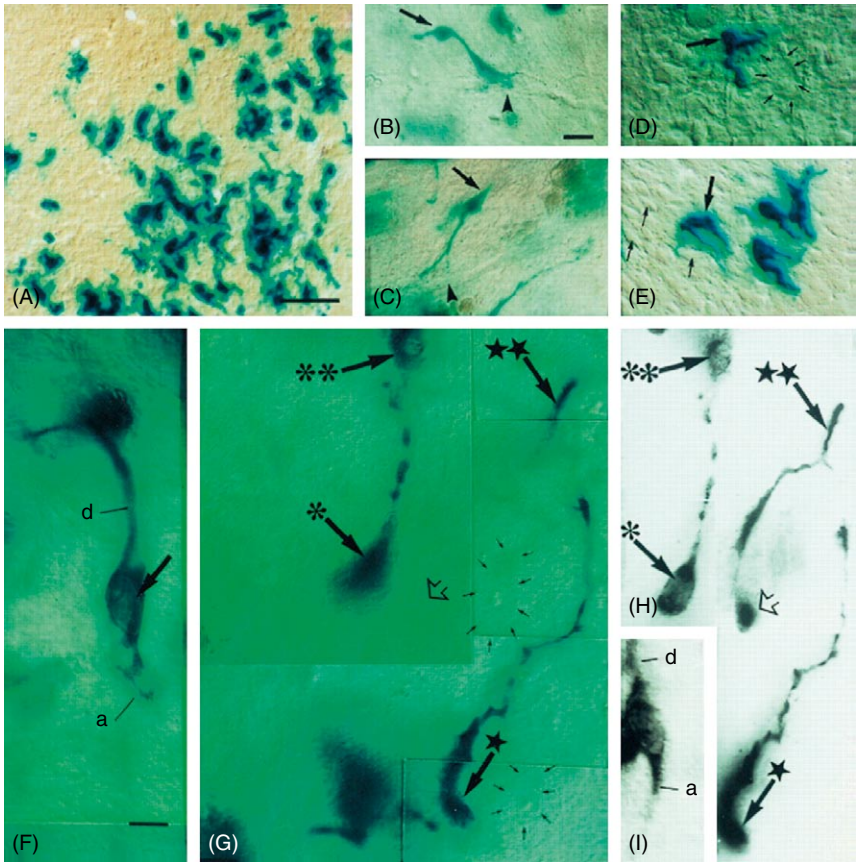


FIGURE 54-3 Multipotent NSCs acquire neuronal morphology in regions of adult neocortex subjected to targeted apoptotic neuronal degeneration. They differentiate into only glia, or remain undifferentiated in intact control cortex. (A) Engrafted X-Gal+ glia at low magnification six weeks following transplantation at 12 weeks; (B–E) higher magnification. (B, C) Donor-derived cells with astroglial features: Small, ovoid cell bodies (arrows) with few, short processes often extending as perivascular end-feet (arrowheads). (D) Small soma of a donor-derived presumptive glial cell (arrow) compared with a much larger (≈30 μm), unlabeled, host pyramidal neuron (small arrows). (E) Donor-derived cells with oligodendroglial features (arrow): Multiprocessed, ensheathing neuronal processes (short arrows). (F–I) A total of 15 ± 7% of engrafted cells in regions of neurodegeneration developed neuronal morphology, resembling pyramidal neurons within layer II/III six weeks following transplantation at 12 weeks. (F, G) Donor-derived cells with neuronal morphology (large arrows): Large somata (20–30 μm diameter) comparable to residual host pyramidal neurons (small arrows in panel G outline two host neurons, visualized under DIC, each with the characteristic large nucleus and prominent nucleolus of a pyramidal neuron), 300–600 μm presumptive apical dendrites positioned between host (small arrows) and donor (large arrows) neurons of similar morphology and size, and presumptive axons. (F) The dark object at the upper end of the presumptive dendrite is another X-Gal+ cell out of the plane of focus. (H) DCM image of panel G, collapsing multiple planes of focus. The cell has the characteristic large nucleus of a pyramidal neuron. All but the terminal dendrite of the soma of the cell is out of the plane of focus in panel G. Cells and neurons are indicated to cross-reference views of the same field in panels G and H. (I) Two overlapping donor-derived pyramidal neurons in different focal planes show a characteristic large nucleus, prominent nucleolus, and axon of the overlying cell and a prominent dendrite of the underlying cell, imaged through multiple focal planes of this thick section under DCM. The identification of the previously-mentioned cells was supported by immunocytochemical and ultrastructural analysis under electron microscopy. In addition, one could determine that donor-derived neurons received synaptic input from the host and were myelinated by host oligodendrocytes, further supporting their incorporation into the host cytoarchitecture (a: axon; d: dendrite; cells: *,★★,** and open arrow; and *: neurons). Bars = 25 μm.

states—that is, diseases in which the pathology is restricted to a particular cell whose lifespan is short-circuited, but within an extracellular milieu otherwise normal. Conversely, a "cell nonautonomous" condition, where normal cells die because of an inhospitable microenvironment, would appear not to be amenable to NSC therapy, given that "replacement" cells would presumably meet a similar fate. Surprisingly, however, NSCs may nevertheless be useful in such cell-extrinsic pathological conditions. It appears that NSCs, particularly in their immature, undifferentiated state, may be more resistant to certain stressors—various toxic metabolites, oxidizing agents—than more mature cells. Furthermore, if the NSCs are not inherently more resistant, they can be readily genetically engineered *ex vivo* to become more resistant.

Despite their extensive plasticity, NSCs never produce cell types inappropriate to the brain (e.g., muscle, bone, or teeth), or yield neoplasms. The use of NSCs as graft material in the CNS may be considered almost analogous to hematopoietic stem cell-mediated reconstitution of the bone marrow. Therefore, the biological repertoire of the NSC, if harnessed, may provide multiple strategies for addressing CNS dysfunction. Some of these approaches have already shown promise experimentally in animal models of neurodegeneration. Some illustrative examples are briefly described in the next sections of this chapter.

GENE THERAPY USING NEURAL STEM CELLS

As stated previously, the ability of NSCs to deliver therapeutic gene products in an immediate, direct, sustained, and perhaps regulated fashion as normal cytoarchitectural components throughout the CNS may overcome some of the limitations of standard viral and cellular vectors (reviewed by Park *et al.*, 2002). The feasibility of this strategy was first demonstrated in a mouse mutant characterized by a single gene defect in all cells, including those in the CNS, leading to their death. The particular mouse modeled the lysosomal storage disease mucopolysaccharidosis type VII (MPS VII), caused by a deletion mutation of the β-glucuronidase (*GUSB*) gene. This incurable inheritable condition is characterized by neurodegeneration in mice, and by progressive mental retardation in humans. Although the particular disease was rare, it served as model for neurological diseases whose etiology stemmed from a genetically-based loss of function. GUSB-secreting NSCs were implanted into the cerebral ventricles of newborn MPS VII mice, allowing the cells access to the subventricular zone (SVZ), a germinal zone from which the cells were disseminated throughout the brain (creating "chimeric forebrains"). These enzyme-producing cells, now in residence as normal cerebral constituents, not only metabolized lysosomal storage normally for themselves, but also cross-corrected mutant cells throughout the brains of recipient mice (Figure 54-1). Employing a similar strategy, retrovirally-transduced NSCs implanted into the brains of fetal and neonatal mice (particularly into periventricular regions) have successfully mediated brain-wide expression of other enzymes—for example, β-hexosaminidase, a deficiency of which leads to the pathological accumulation of GM_2 ganglioside.

Findings such as these have helped to establish the paradigm of using NSCs for the transfer of other factors of therapeutic or developmental interest into and throughout the CNS. Although NSCs express baseline amounts of particular enzymes and neuroprotective factors, NSCs can be genetically-modified to enhance their production of these molecules, or to produce additional molecules that might enhance their therapeutic potential. For example, NSC have been used to deliver NT-3 within the hemisectioned rat spinal cord, to express NGF and BDNF within the septum and basal ganglia, to provide tyrosine hydroxylase to the parkinsonian striatum, and to express myelin basic protein (MBP) throughout the dysmyelinated cerebrum. In one illustrative set of experiments, NSCs that overexpressed NGF were transplanted into the striatum of a rat lesioned with quinolinic acid, a toxin used to emulate some of the neuronal loss seen in Huntington's disease. Delivery of NGF by the engrafted NSCs appeared to reduce the size of the lesion, and to promote sparing of host striatal neurons. When implanted into the septum of aging rats, NGF-overexpressing NSCs appeared to blunt the typical cognitive decline by preventing age-related atrophy of forebrain cholinergic neurons. Well-integrated into the host tissue, the engrafted NSCs continued to produce NGF until nine months after grafting.

That NSCs appear to have a strong affinity for sites of pathology, and will migrate extensive distances (even from the opposite cerebral hemisphere) to home in on them, makes the NSC unique and valuable as a gene delivery vehicle. For example, a type of pathology particularly elusive to gene therapeutic interventions has been the brain tumor, especially the glioma. Gliomas are so exceptionally migratory and infiltrative that they elude even the most effective surgical, radiation, or gene therapeutic strategy. However, the ability of transgene-expressing NSCs to "track down" and deliver therapeutic gene products directly to these widely-dispersed and invasive neoplastic cells makes them a potentially valuable adjunct in the treatment of these aggressive tumors.

Although this therapeutic approach—exploiting the normal biological behavior of NSCs for transplantation-based gene therapy—is being extended to animal models of many neurological disorders, it is important to recognize that each pathophysiological process, each animal model, and each therapeutic molecule must be assessed and optimized individually.

CELL REPLACEMENT USING NEURAL STEM CELLS

It is postulated that NSCs persist within the CNS long beyond cerebrogenesis to maintain homeostasis following perturbations in the CNS. Presumably, the mechanisms that allow the endogenous NSC to perform this function are preserved when the cells are isolated from the CNS, expanded in culture, and reimplanted into the damaged CNS. Indeed, it appears that when NSCs are so implanted, they respond by shifting their pattern of differentiation toward replenishing the missing cell type.

The study that first demonstrated this phenomenon was performed on a model of experimentally-induced apoptosis of selectively targeted pyramidal neurons in the adult mammalian neocortex. When transplanted into this circumscribed, neuron-depleted region, 15% of the NSCs altered the differentiation path they would otherwise have pursued in the intact adult, non-neurogenic mammalian neocortex (i.e., to become glia); instead, they differentiated specifically into pyramidal neurons, partially replacing that lost neuronal population (Figure 54-3). Of these, a sub-population spontaneously sent axonal projections to their proper targets in the contralateral cortex. Outside the borders of this small region of selective neuronal death, the NSCs yielded only glia. Thus, neurodegeneration appeared to create a milieu that recapitulated embryonic development cues (e.g., for cortical neurogenesis), and the NSCs were sufficiently sensitive to detect and respond to this micromolecular alteration—possibly to therapeutic advantage.

Apoptosis is implicated in a growing number of both neurodegenerative and normal developmental processes. Whether this differentiation shift occurs only in response to signals associated with apoptosis, or in response other types of cell death as well, remains to be determined. Most neurological diseases are characterized by a mixture of neuropathological processes, making dissection of the key stimuli complex. For example, we have observed that, following an ischemic insult, NSCs will robustly repopulate infarcted regions of the postdevelopmental cortex, and differentiate into cortical neurons in these regions. However, even hypoxic–ischemic cerebral injury—the quintessential example of necrotic and excitotoxic injury—is characterized by an apoptotic phase. An added level of complexity may be the tempo at which apoptotic signals are elaborated—an acute burst may be more instructive than a more languid burst.

Studies are ongoing to identify the key molecules that direct NSC fate during and following various kinds of neural injury. Intriguingly, we are starting to learn that cytokines released during an inflammatory reaction—those emanating from microphages and microglia, as well as from parenchymal damage (e.g., SDF1-α)—play a pivotal role in "beckoning" NSCs. Coincidentally, we are starting to recognize that many processes regarded as neurodegenerative—including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, tumors, and stroke—are characterized by a prominent inflammatory signature. Although each is different in its etiology, region of involvement, and course, inflammation may be their common denominator from the "viewpoint" of the NSC.

"GLOBAL" CELL REPLACEMENT USING NEURAL STEM CELLS

The targeted-apoptosis model described in the previous section exemplifies a circumscribed type of neural cell loss. However, the pathologic lesions of many neurological disorders are often widely—even globally—dispersed throughout the brain. Such conditions include the neurodegenerative disorders of childhood (e.g., inborn errors of metabolism, storage diseases, leukodystrophies, and neuronal ceroid lipofuscinoses) and hypoxic–ischemic encephalopathy, as well as some adult CNS diseases (e.g. multiple sclerosis, Alzheimer's disease, and ALS). Treatment for these disorders requires widespread replacement of genes, cells, or both, as well as the regeneration, protection, or both, of broad networks of neural circuitry. The ability of inherently migratory NSCs to integrate into germinal zones from which they can be "launched," and the inclination of NSCs to travel long distances to home in on pathologic regions, makes these cells ideally—and perhaps uniquely—suited for this task.

Mouse mutants characterized by CNS-wide white matter disease provided the first models for testing the hypothesis that NSCs might be useful against neuropathologies requiring widespread neural cell replacement. The oligodendrocytes—the myelin-producing cells—of the dysmyelinated *shiverer (shi)* mouse are dysfunctional because they lack MBP, a molecule essential for effective myelination. Therapy, therefore, requires widespread replacement with MBP-expressing oligodendrocytes. NSCs transplanted at birth (employing the intracerebroventricular implantation technique described previously for the diffuse engraftment of enzyme-expressing NSCs to treat global metabolic lesions) resulted in engraftment throughout the *shi* brain with repletion of significant amounts of MBP. Of the many donor cells that differentiated into oligodendrocytes, a subgroup myelinated ~40% of host neuronal processes. In some recipient animals, the symptomatic tremor decreased (Figure 54-4). Therefore, "global" cell replacement was shown to be feasible for some pathological conditions if cells with stem-like features are employed. This approach has been extended to other myelin-impaired animal models, such as the experimental allergic encephalomyelitis mouse model of multiple sclerosis, as well as rodent mutant models of Palezeus-Merzbacher, Krabbe, and Canavan leukodystrophies. Recently, it was demonstrated that neural progenitor stem cells of human origin have a similar remyelinating capacity in the *shi* mouse

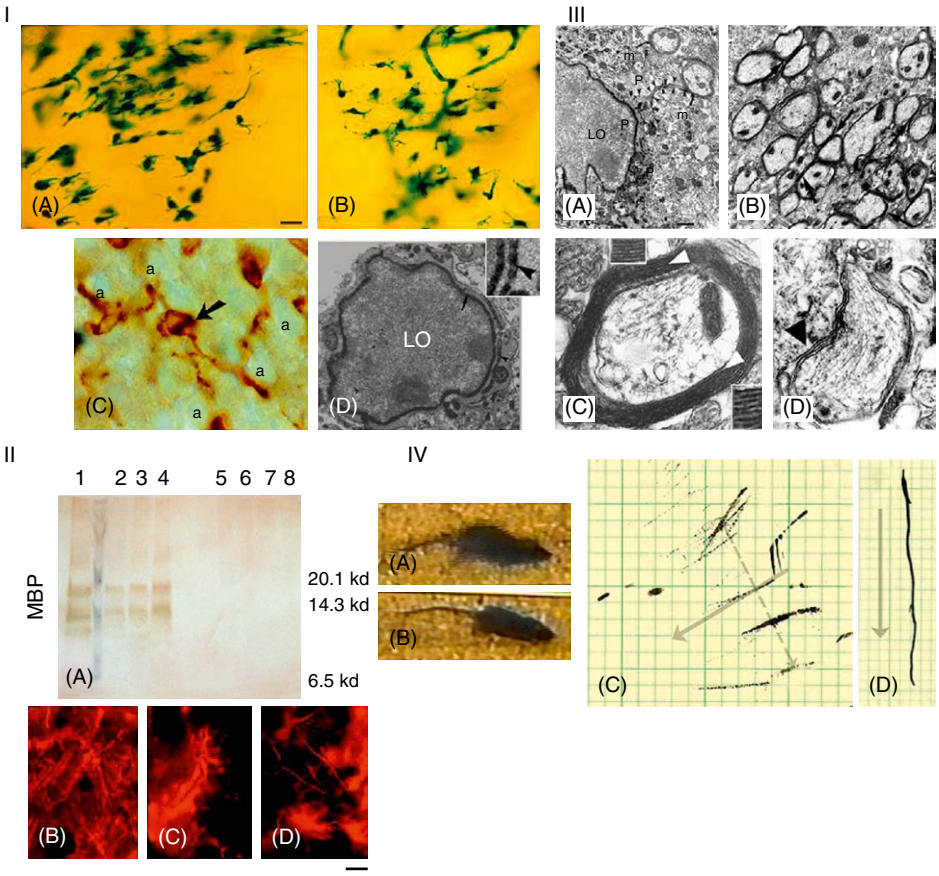


FIGURE 54-4 "Global" cell replacement is feasible using NSC transplantation: evidence from the dysmyelinated *shi* mouse brain. (A) NSCs engraft extensively throughout the *shi* dysmyelinated brain, including within white tracts, and differentiate into oligodendrocytes. *LacZ*-expressing, βgal-producing NSCs were transplanted into newborn *shi* mutants and analyzed systematically at intervals between two and eight weeks following engraftment. Coronal sections through the *shi* brain at adulthood demonstrated widely-disseminated integration of Xgal+ donor-derived cells throughout the neuraxis, similar to the pattern seen in Figure 54-1A in the MPS VII mutant mouse. (1 and 2) Donor-derived Xgal+ cells (higher magnification) in sections through the corpus callosum possessed characteristic oligodendroglial features (small, round, or polygonal cell bodies with multiple fine processes oriented toward the neural fiber tracts). (3) Close-up of a donor-derived anti-β immunoreactive oligodendrocyte (arrow) extending multiple processes toward, and beginning to enwrap, large, adjacent axonal bundles (a) viewed on end in a section through the corpus callosum. That cells such as those in panel A 1–3 and in panel B 2–4 were oligodendroglia was confirmed by the electron micrograph in panel A 4 and in panel C, demonstrating their defining ultrastructural features. A donor-derived Xgal+ oligodendrocyte (LO) can be distinguished by the electron dense Xgal precipitate, typically localized to the nuclear membrane (arrow), ER (arrowhead), and other cytoplasmic organelles. The ER is magnified in the inset to demonstrate the unique crystalline nature of individual precipitate particles. (B) MBP expression in mature transplanted and control brains. (1) Western analysis for MBP in whole brain lysates. The brains of three transplanted *shi* mutants (lanes 2–4) expressed MBP at levels close to those of an age-matched unaffected mouse (lane 1, positive control) and significantly greater than the amounts seen in untransplanted (lanes 7–8, negative control) or unengrafted (lanes 5–6, negative control) age-matched *shi* mutants. (Identical total protein amounts were loaded in each lane.) (2–4) Immunocytochemical analysis for MBP. (2) The brain of a mature unaffected mouse was immunoreactive to an antibody to MBP (revealed with a Texas red-conjugated secondary antibody). (3 and 4) Age-matched engrafted brains from *shi* mice similarly showed immunoreactivity. Because untransplanted *shi* brains lack MBP, MBP immunoreactivity has classically been a marker for normal donor-derived oligodendrocytes in transplant paradigms. (C) NSC-derived "replacement" oligodendrocytes are capable of myelination of *shi* axons. In regions of MBP-expressing NSC engraftment, *shi* neuronal processes became enwrapped by thick, better-compacted myelin. (1) Two weeks after transplant, a donor-derived, labeled oligodendrocyte (LO), recognized by extensive Xgal precipitate (p) in the nuclear membrane, cytoplasmic organelles, and processes, was extending processes (arrowheads) to host neurites, and was beginning to ensheathe them with myelin (m). (2) If engrafted *shi* regions (1) were followed to four weeks, the myelin began to appear healthier, thicker, and better-compacted (arrows) than that in age-matched untransplanted control mutants. (3) By six weeks after transplant, these matured into even thicker wraps; ~40% of host axons were ensheathed by myelin (white arrowheads; MDLs are evident) that was dramatically thicker and better-compacted than that of *shi* myelin (4, black arrowhead) from an unengrafted region of an otherwise successfully engrafted *shi* brain). (D) Functional and behavioral assessment of transplanted *shi* mutants and controls. The *shi* mutation is characterized by the onset of tremor and a "shivering gait" by postnatal week two or three. The degree of motor dysfunction in animals was gauged in two ways: by blindly scoring periods of standardized, videotaped cage behavior of experimental and control animals; and by measuring the amplitude of tail displacement from the body's rostral–caudal axis (an objective, quantifiable index of tremor). Video freeze-frames of (4.4a) unengrafted and (4.4b) successfully engrafted *shi* mice. (4.4a) The whole body tremor and ataxic movement observed in the unengrafted symptomatic animal causes the frame to blur, a contrast with (1) the well-focused frame of the asymptomatic transplanted *shi* mouse. Of transplanted mutants, 60% evinced nearly normal-appearing behavior (2) and attained scores similar to normal controls. (3 and 4) Whole body tremor was mirrored by the amplitude of tail displacement (dotted gray arrow), measured perpendicularly from a line drawn in the direction of the animal's movement. Measurements were made by permitting a mouse, whose tail had been dipped in Indian ink, to move freely in a straight line on a sheet of graph paper. (3) Large degrees of tremor cause the tail to make widely-divergent ink marks from the midline, representing the body's axis (solid gray arrow). (4) Absence of tremor allows the tail to make long, straight, uninterrupted ink lines on the paper congruent with the body's axis. The distance between points of maximal tail displacement from the axis was measured and averaged for transplanted and untransplanted *shi* mutants and for unaffected controls (dotted gray arrow). Panel 3 shows data from a poorly-engrafted mutant that did not improve with respect to tremor, whereas panel 4 reveals lack of tail displacement in a successfully-engrafted asymptomatic mutant. Overall, 64% of transplanted *shi* mice examined displayed at least a 50% decrement in the degree of tremor or "shiver." Several showed no displacement.

brain. Viewed more parochially, the ability of NSCs to remyelinate is of significant importance, because impaired myelination plays such an important role in many genetic (e.g., leukodystrophies and inborn metabolic errors) and acquired (e.g., traumatic, infectious, asphyxial, ischemic, and inflammatory) neurodegenerative processes. However, viewed more broadly, the ability of the NSC to replace this particular type of neural cell throughout the brain bodes well for its ability to replace other classes of neural cell across the broad terrains often demanded by other categories of complex neurodegenerative diseases.

NEURAL STEM CELLS DISPLAY AN INHERENT MECHANISM FOR RESCUING DYSFUNCTIONAL NEURONS

The examples cited previously highlight that an abnormal environment can direct the behavior of a grafted NSC. However, they leave the impression that the exogenous NSC alone fills gaps. The situation is more complex and richer. We are beginning to learn that the NSC and the injured host engage in a dynamic series of ongoing reciprocal interactions, each instructing the other. Under instruction from exogenous NSCs, the injured host nervous system also contributes to its own repair. These important stem cell phenomena were first illustrated in a few examples.

The effect of NSCs in directly rescuing endangered host neurons was first evinced in a series of experiments in aged rodents in which the nigrostriatal system was impaired (Figure 54-5). Parkinson's disease is a degenerative disorder characterized by a loss of midbrain dopamine (DA) neurons, with a subsequent reduction in striatal DA. The disease, in addition to incapacitating thousands of patients, has long served as a model for testing neural cell replacement strategies. Transplantation therapy for this CNS disorder has a long history (for a review, see Isacson *et al.*). It was the neural disease first treated clinically by neural transplantation, using primary tissue from human fetal ventral mesencephalon to replace DA-expressing cells. Indeed, in this disease, the limitations of fetal tissue grafts not only in rodent and primate models of Parkinson's disease, but also in clinical trials, were first recognized. These limitations include, on the one hand, short graft survival and limited integration of the grafts and, on the other hand, the possibility of unregulated DA production in improper regions, leading adversely to dyskinesias. Given the storied role of Parkinson's disease in the development of cellular therapies, it is appropriate that a model of this disease should also have played a pivotal role in revealing a little-suspected but powerful therapeutic action that NSCs may play in preserving degenerating host cells by some heretofore unheralded mechanisms that are nevertheless inherent to stem cell biology.

In the hope that NSCs might spontaneously differentiate into DA neurons when implanted into a DA-depleted region of the CNS, unmanipulated murine NSCs were implanted unilaterally into the substantia nigra of aged mice exposed systemically to high-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that produces a persistent impairment of mesencephalic DA neurons and their striatal projections. The NSCs not only migrated from their point of implantation and integrated extensively within both hemispheres, but also were associated with a dramatic reconstitution of DA function throughout the mesostriatal system. Although there was spontaneous conversion of a sub-population of donor NSCs into dopaminergic neurons in DA-depleted areas, contributing to nigral reconstitution, most (80–90%) dopaminergic neurons in the "reconstituted midbrain" were actually host cells that had been "rescued" by factors produced constitutively by the NSCs with which they were juxtaposed and that had not become neurons. These chaperone cells constitutively produce substantial amounts of neurosupportive agents. One such prominent molecule was GDNF, a factor known to be neuroprotective of ventrally located neurons (including DA neurons and spinal–ventral horn cells). A similar observation is beginning to emerge from the implantation of human NSCs into the MPTP-lesioned, subhuman primate model of Parkinson's disease.

A sense of the extent of the cross talk also became evident when examining rodent models of hypoxia–ischemia, a common cause of neurological disability in adults and children. Hypoxia–ischemia causes much of its damage from extensive loss of cerebral parenchyma, and the cells and connections that reside there. When NSCs are implanted into these regions of extensive degeneration (particularly when transiently supported by biodegradable scaffolds), robust reciprocal interactions ensue spontaneously between the exogenous implant and the injured host brain, substantially reconstituting parenchyma and anatomical connections, as well as reducing parenchymal loss, secondary cell loss, inflammation, and scarring. Similar results are observed in the hemi-resectioned adult rodent spinal cord, in which evidence of an up-regulated host neuronal regenerative response is noted, resulting in significant functional improvement.

Indeed, the ability of engrafted NSCs to exert a protective and regenerative influence on degenerating host neural systems, because of their intrinsic expression of trophic factors, is being observed in an increasing number of conditions. For example, the implantation of murine and human NSCs into the spinal cords of the SOD1 transgenic mouse model of ALS, a disease characterized by virulent motor neuron degeneration, has been pivotal in protecting these ventral horn cells from death, preserving motor and respiratory function, blunting disease progression, and extending life. NSCs can similarly protect other neuron pools, promote motor axonal outgrowth following traumatic spinal cord injury, preserve infarcted regions of

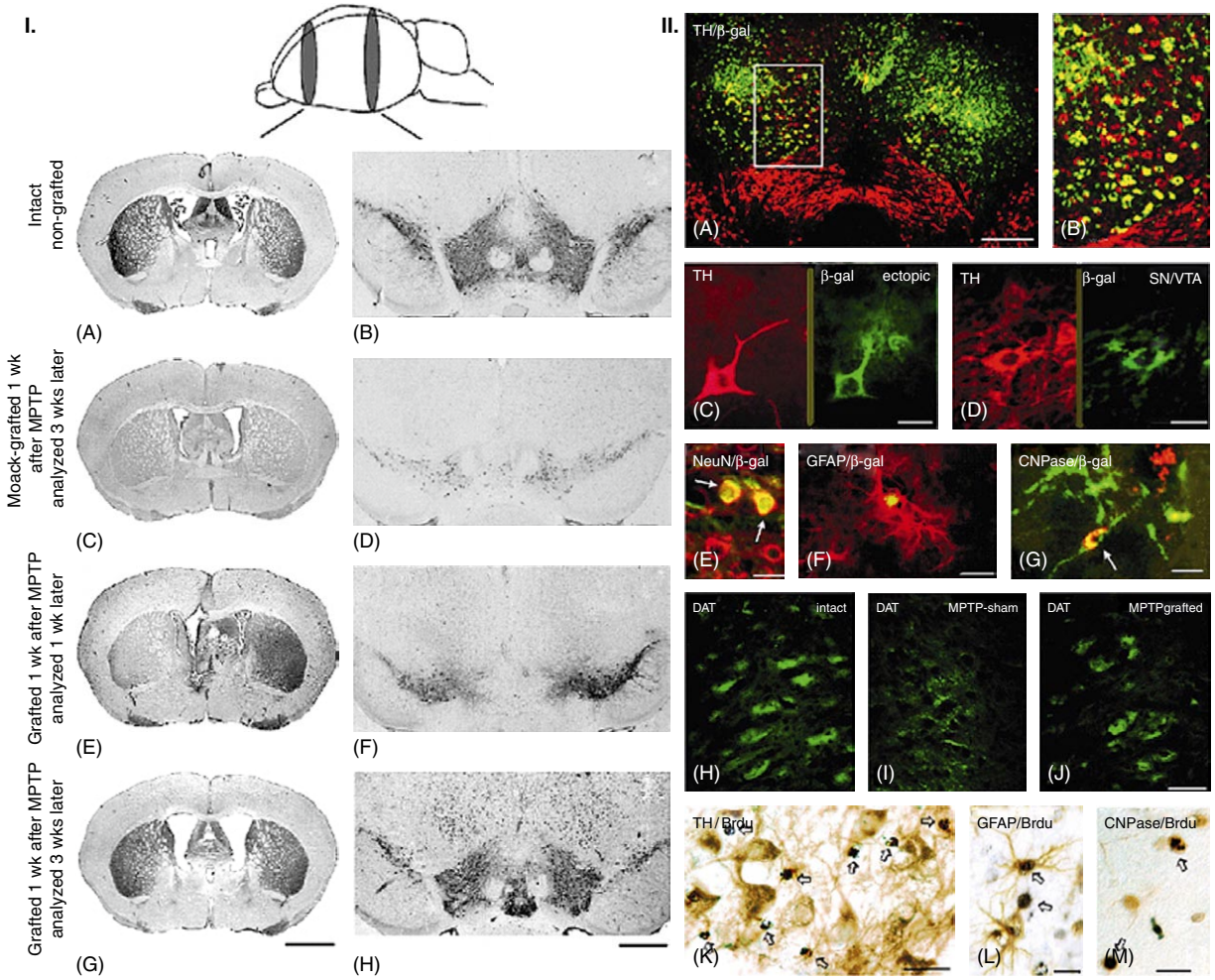


FIGURE 54-5 NSCs possess an inherent mechanism for rescuing dysfunctional neurons: evidence from the effects of NSCs in the restoration of mesencephalic dopaminergic function. (A) TH expression in mesencephalon and striatum of aged mice following MPTP lesioning and unilateral NSC engraftment into the substantia nigra–ventral tegmental area (SN–VTA). A model that emulates the slow dysfunction of aging dopaminergic neurons in SN was generated by giving aged mice repeated high doses of MPTP. Schematic indicates the levels of the analyzed transverse sections along the rostrocaudal axis of the mouse brain. Coronal sections are through the striatum in the left column and through the SN–VTA area in the right column. Immunodetection of TH (black cells) shows the normal distribution of DA-producing TH+ neurons in: (2) coronal sections in the intact SN–VTA; and (1) their projections to the striatum. Within one week, MPTP treatment caused extensive and permanent bilateral loss of TH immunoreactivity in both: (3) the mesostriatal nuclei; and (4) the striatum. Shown in this example, and matching the time point in 7 and 8, is the situation in a mock-grafted animal four weeks after MPTP treatment. Unilateral (right side) stereotactic injection of NSCs into the nigra is associated, within one week after grafting, with substantial recovery of TH synthesis within: (6) the ipsilateral DA nuclei; and (5) their ipsilateral striatal projections. By three weeks after transplant, however, the asymmetric distribution of TH expression disappeared, producing TH immunoreactivity in: (8) the midbrain; and (7) striatum of both hemispheres that approached the immunoreactivity of (1 and 2) the intact controls, and gave the appearance of mesostriatal restoration. Similar observations were made when NSCs were injected four weeks after MPTP treatment (not shown). Bars: 2 mm (left), 1 mm (right). Note the ectopically placed TH+ cells in panel 8. These are analyzed in greater detail with the entire SN in B. (B) Immunohistochemical analyses of TH, DAT, and BrdU+ cells in MPTP-treated and grafted mouse brains. The initial presumption was that the NSCs had replaced the dysfunctional TH neurons. However, examination of the reconstituted SN with dual βgal and TH ICC showed that: (1 and 3) 90% of the TH+ cells in the SN were rescued, host-derived cells; and (4) only 10% were donor-derived cells. Most NSC-derived TH+ cells were just above the SN ectopically (blocked area in panel 1; enlarged in panel 2). These photomicrographs were taken from immunostained brain sections from aged mice exposed to MPTP, transplanted one week later with NSCs and sacrificed after three weeks. The following combinations of markers were evaluated: (1–4) TH with βgal; (5) NeuN with βgal; (6) GFAP with βgal; (7) CNPase with βgal; (8) TH with BrdU; (9) GFAP with BrdU; and (10) CNPase with BrdU. Anti-DAT-stained areas are revealed in the SN of: (11) intact; (12) mock-grafted; and (13) NSC-grafted brains. Fluorescence filters for Alexa Fluor 488 and Texas red and a double-filter for both types of fluorochromes were used to show antibody binding. (3, 4, and 8–10) Single-filter exposures; (1, 2, and 5–7) double-filter exposures. (1) Low-power overview of the SN–VTA of both hemispheres. Most TH+ cells within the nigra are of host origin (~90%), a much smaller proportion (~10%) are donor derived (close-up in panel 4). Although a significant proportion of NSCs differentiated into TH+ neurons, many of these resided ectopically, dorsal to the SN (boxed area in panel 1 enlarged in panel 2; and a high-power view of the donor-derived cell that was also TH+ in panel 3), where the ratio of donor-to-host cells was inverted: ~90% donor derived compared with ~10% host derived. Note the near absence of a βgal-specific signal in the SN–VTA, whereas ectopically, many of the TH+ cells were double labeled and thus NSC derived. (5) NSC-derived non-TH neurons (NeuN+, arrow); (6) astrocytes (GFAP+); and (7) oligodendrocytes (CNPase+, arrow) were also seen, both within the mesencephalic nuclei and dorsal to them. (10) The DAT-specific signal suggests that the reconstituted mesencephalic nuclei in the NSC-grafted mice were functional DA neurons comparable to those seen (8) in intact nuclei, but not (9) in MPTP-lesioned, sham-engrafted controls. This further suggests that the TH+ mesostriatal DA neurons affected by MPTP are functionally impaired. Note that (9) sham-grafted animals contain only punctate residual DAT staining within their dysfunctional fibers, whereas DAT staining (8) in normal and (10) in engrafted animals was normally and robustly distributed, both within processes and throughout their cell bodies. (11–12) Any proliferative BrdU+ cells after MPTP insult, graft, or both were confined to glial cells, whereas (11) the TH+ neurons were BrdU–. This finding suggested that the reappearance of TH+ host cells was not the result of neurogenesis, but rather the recovery of extant host TH+ neurons. Bars = 90 μm (1), 20 μm (3–5), 30 μm (6), 10 μm (7), 20 μm (8–10), 25 μm (11), 10 μm (11), and 20 μm (12).

cerebrum, induce vascularization of reconstituted regions of cortical parenchyma, and inhibit inflammation and scarring following traumatic or ischemic insult.

Together, these observations suggest that exogenous NSCs may not only replenish inadequate pools of endogenous NSCs to compensate for missing neural cells, but also reactivate or enhance endogenous regenerative and protective capacities. It also highlights a heretofore unanticipated mechanism by which NSCs may exert a therapeutic effect—to be added to their more traditional roles in direct cell replacement and gene therapy.

NEURAL STEM CELLS AS THE GLUE THAT HOLDS MULTIPLE THERAPIES TOGETHER

Experimentally, one may eliminate a particular cell type, lesion a particular region of CNS, knockout a particular gene, or choose a mouse strain in which, by chance, certain mutations have spontaneously occurred. However, most human neurodegenerative diseases are not as "clean." They are quite complex. And complex diseases, such as those affecting the nervous system, will require complex and multifaceted solutions—including pharmacological, gene and molecular, cell replacement, tissue engineering, angiogenic, anti-inflammatory, antiapoptotic, pro-regenerative, and pro-neurite outgrowth-promoting therapies. The NSC, as a key player in a set of fundamental developmental mechanisms, may serve as the glue that holds many of these strategies together. How they may be intelligently, effectively, safely, and practically orchestrated in actual patients will require a good deal of careful investigation.

For example, as our sophistication about disease processes grow, we are beginning to learn that more than one neural cell type probably needs to be replaced in a disease. For example, in a disease like ALS, a disorder characterized by progressive motor neuron degeneration, we are beginning to learn that astrocyte replacement may be just as critical as motor neuron replacement. Conversely, in multiple sclerosis, a white matter disease characterized by oligodendrocyte degeneration, replacing neurons and their axonal connections may be critical for the restoration of function. These same caveats may apply to many diseases in which replacement of multiple cell types may be the key to neurological reconstitution and to damaged milieu reconstruction. Because of their ability to develop into multiple integral, cytoarchitectural components of many regions throughout the host brain, NSCs may be able to replace a range of missing or dysfunctional neural cell types within the same region. This is important in the likely situation in which return of function may require the reconstitution of the milieu of a given region (e.g., not just the neurons, but also the chaperone cells—the glia and support cells) to nurture, detoxify, guide, and/or myelinate the neurons. As noted previously, the NSCs, especially in particular differentiation states, express certain genes of intrinsic interest (many neurotrophic factors, lysosomal enzymes, angiogenic factors, anti-inflammatory molecules, antioxidants, etc.), or they can be engineered *ex vivo* to do so. The challenge remains, however, to coordinate these multifaceted therapies so that they work in concert synergistically, and not at cross-purposes.

SUMMARY

The ability of NSCs to migrate and integrate throughout the brain, as well as to disseminate a foreign gene product, is of great significance for the development of new therapies for neurodegenerative diseases in humans. Lethal, hereditary, neurodegenerative diseases of childhood, such as the gangliosidoses, leukodystrophies, neuronal ceroid lipofuscinoses, and other storage diseases, result in lesions throughout the CNS. Diseases of adult onset (e.g., Alzheimer's disease) are also diffuse in their pathology. Even acquired diseases, such as spinal cord injury, head trauma, and stroke, are broader in their involvement than typically assumed. Such abnormalities may benefit from the multifaceted approach NSCs may enable. First, they may replace a range of cell types—not only neurons to reconstitute neural connections, but also oligodendrocytes to elaborate myelin and astrocytes to serve trophic, guidance, protective, and detoxification functions. Second, NSCs may deliver exogenous genes that might restore normal metabolism, complement a factor deficiency, support the survival of a damaged host neuron, neutralize a hostile milieu, counteract a growth-inhibitory environment, promote neurite regrowth, and reform stable and functional contacts. As noted previously, many molecules are produced inherently by NSCs when they are in particular states of differentiation. The production of other molecules might require *ex vivo* genetic engineering. NSCs appear to have their greatest therapeutic effect when used in the early stages of a degenerative process, or in the subacute phase following injury.

A major requirement for the better use of NSCs will be a better understanding of the pathophysiology of the diseases to be targeted, i.e., knowing what aspects require repair, and which cell type or types require replacement or rescue. Another challenge will be determining how to exploit stem cell biology for chronic conditions—in other words, how the acute milieu can be recreated in the chronic environment such that NSCs behave therapeutically there as well.

Among the methodological hurdles will be to devise how, when, where, and with what frequency to deliver NSCs to adults with disseminated diseases in regions not fed by readily accessible germinal zones, e.g., the spinal cord in which the central canal is no longer patent. Linked to this is the need for a better understanding of the methods

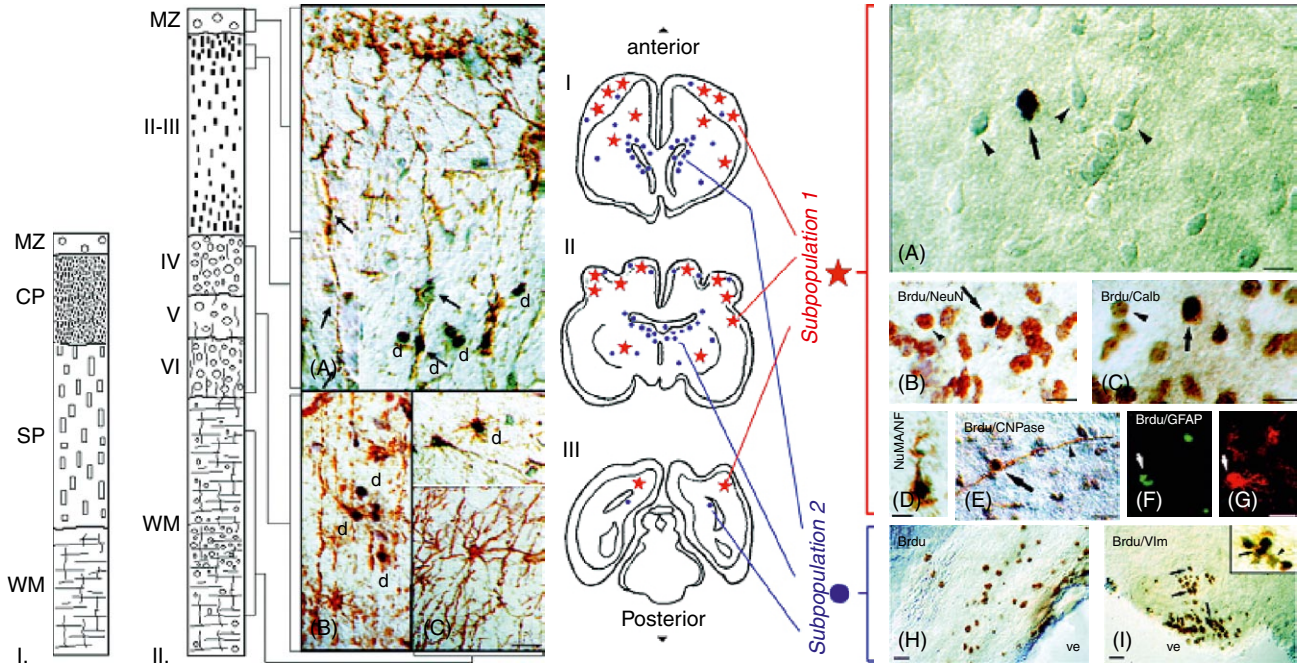


FIGURE 54-6 Clonal human NSCs migrate from the VZ into the developing neocortex. Schematics of the developing monkey neopallium (A) at the time of transplantation. (1) 12–13 wpc and (2) at the time of death (16–17 wpc). (3–5) Photomicrographs from selected locations spanning the neopallium. (Their location relative to the schematic is indicated by brackets.) (3) Injected into the left lateral ventricle and having integrated throughout the VZ, the human NSC-derived cells (d), identified by their BrdU immunoreactivity (black nuclei), migrated along the monkey's radial glial processes through the neopallial wall, to reach their temporally appropriate destination in the nascent superficial layers II and III (3), where they detached from the radial glia and took up residence as neurons. Arrows indicate climbing (donor- and host-derived) cells positioned along the processes of the vimentin-positive host radial glia. Some cells (inset) are still attached to these fibers and in the process of migration. (4 and 5) Immature, donor, human NSC-derived astrocytes intermixed with host-derived astrocytes in deeper cortical lamina, having differentiated as expected for that site and time. (B) Segregation of the fates of human NSCs and their progeny into two sub-populations in the brains of developing Old World monkeys. Schematics (left) and photomicrographs (right) illustrate the distribution and properties of clonal human NSC-derived cells. Human NSCs (labeled with BrdU) dispersed throughout and integrated into the VZ. From there, clonally-related human NSC-derived cells pursued one of two fates. Those donor cells that migrated outward from the VZ along radial glial fibers into the developing neocortex constituted one pool or sub-population. (4–12) The differentiated phenotypes of cells in this sub-population (1) (stars in the schematic), particularly in layers II and III. (4) A human NSC-derived BrdU+ cell (black nucleus, arrow)—likely a neuron according to its size, morphology, large nucleus, and location—is shown (under Nomarski optics) intermingled with the monkey's similar neurons (arrowheads) in neocortical layers II and III. The neuronal identity of such donor-derived cells is confirmed by immunocytochemical analysis. High-power photomicrographs of human donor-derived cells integrated into the monkey cortex double-stained with antibodies against BrdU and cell type-specific markers: (5) NeuN; and (6) calbindin for neurons (arrows, donor-derived cells; arrowheads, host-derived cells). (8) CNPase for oligodendroglia (arrow). (BrdU+ is the black nucleus in the CNPase+ cell; the arrowhead indicates the long process emanating from the soma.) (9, 10) GFAP for astroglia antibody to BrdU revealed through fluorescein; (9) antibody to GFAP revealed through Texas red. The human origin of the cortical neurons is independently confirmed in panel 7, where the human-specific nuclear marker NuMA (black nucleus) is colocalized in the same cell with neurofilament immunoreactivity. Progeny from this same human NSC clone were allocated to a second cellular pool—sub-population 2 (dots in the schematic; arrows in panels 9 and 10)—that remained mainly confined to the SVZ, and stained only for an immature neural marker. (Vimentin colocalized with BrdU is easily seen in inset (arrows); the arrowhead indicates the host vimentin-positive cell.) Some members of sub-population 2 were identified within the developing neocortex (dots) intermixed with differentiated cells. Panels 9 and 10 use immunofluorescence; the other immunostains use a DAB-based color reaction. Scale bars = 30 μm (4–6) and 20 μm (7–12). (d: human NSC-derived cells, MZ: marginal zone, CP: cortical plate, SP: subplate, WM: white matter, and II–VI: cortical layers).

for augmenting, yet controlling, the propagation and the phenotype specification of NSCs *in vitro* and *in vivo*.

Another question has come to dominate the stem cell field: what is the most effective way to obtain NSCs for therapy? Should they be obtained directly from regions of neuroectodermal origin, and if so, should that be from a fetal source? Can an adult source be equally effective? Should stable lines of NSCs be established that can be used for all patients, or should NSCs be abstracted from each patient to be used as an autograft on a case-by-case basis? The degree to which the immune system presents a barrier to stem cell transplantation will likely determine the answers to this question and its subquestions. If ES cells (from the inner cell mass of blastocysts) are directed to become NSCs *in vitro*, will such cells be equally as safe and effective?

The success in isolating stem-like cells from the CNS (the first "solid organ stem cells" discovered), and their therapeutic potential, also gave rise to the search for and successful isolation of stem-like cells from other non-neural organ systems, including bone marrow mesenchyme, muscle, skin, retina, and liver, for the purposes of repairing those tissues. Whether such *non-neural* stem cells can

yield *neural* stem cells—through metaplasia or transdifferentiation—remains unresolved and exceptionally controversial. Can NSCs be derived efficiently and effectively from non-neural organs with outcomes as good as from NSCs themselves?

A better understanding of fundamental NSC biology will be required before human NSCs can be transplanted efficaciously in true clinical settings. Nevertheless, progress in this regard is being made. Several NSC lines have been established from the human fetal telencephalon and spinal cord that seem to emulate many of the appealing properties of their rodent counterparts: They differentiate *in vitro* and *in vivo* into neurons, astrocytes, and oligodendrocytes; they follow appropriate developmental programs and migrational pathways similar to endogenous precursors following engraftment into developing rodent and subhuman primate brain (Figure 54-6); they express foreign genes *in vivo* in a widely-disseminated manner; and they can replace missing neural cell types when grafted into various mutant mice or rodent models of injury. The principal differences between human NSCs and mouse NSCs so far seem to be the length of the cell cycle (up to four days in the human), and the predilection of human NSCs to senesce (after ~100 cell divisions), obstacles that are being actively addressed. If human NSCs behave in lesioned subhuman primate brains, with safe and effective engraftment and foreign gene expression, as they seem to do in rodents, then human trials might be warranted for testing their value against certain genuine clinical neurodegenerative diseases. Through a careful and circumspect series of experiments and trials, we may learn whether we, indeed, have found within Nature's own toolbox a powerful and versatile therapeutic tool—one of the goals of experiments that started more than 15 years ago.

KEY WORDS

Acute degeneration Damage is restricted to a circumscribed region (both in space and time), multiple neural cells types must be replaced (for example stroke, CNS injury, and spinal injury).

Chronic degeneration Progressive neurodegenerative diseases requiring the replacement of all neural cell types in large brain regions, in the face of ongoing degeneration and possibly the presence of toxic molecules.

Demyelination disorders Mainly of two classes; disorders in which endogenous myelinating cells are genetically impaired and fail to myelinate during development, and disorders that result from loss/failure of remyelination in the adult (for example, multiple sclerosis, allergic encephalomyelitis, and injury).

Neural stem cells (NSCs) NSCs are the most primordial cells of the nervous system. They generate the array of specialized cells throughout the CNS (and probably the peripheral, autonomic, and enteric nervous systems as well). NSCs are operationally defined by their ability: (1) to differentiate into cells of all neural lineages (i.e., neurons, ideally of multiple subtypes; oligodendroglia, and astroglia) in multiple regional and developmental contexts; (2) to self-renew; and (3) to populate developing and/or degenerating CNS regions.

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Chapter 55

Spinal Cord Injury

John W. McDonald and Daniel Becker

INTRODUCTION

Spinal cord injury (SCI) is a major medical problem, because there is currently no way to repair the central nervous system (CNS) and restore function. In this chapter, we focus on embryonic stem (ES) cells as an important research tool and potential therapy. We review studies that have used ES cells in spinal cord repair. We conclude that progress has been good, that knowledge is still too limited, and that harnessing the potential of ES cells will be important for solving the problem of SCI.

PROBLEM

Nearly 12 000 people suffer a traumatic SCI every year, and about a quarter of a million Americans are living with this devastating condition. There are also four to five times as many spinal cord injuries caused by medical conditions, such as multiple sclerosis, ALS (Lou Gehrig's disease), polio, HTLV-1, metabolic deficits, stenosis, and disc herniation.

The consequences of SCI depend on the level at which the cord is damaged. In its severest form, SCI causes paralysis and loss of sensation throughout the body, inability to control bowel and bladder function, trouble controlling autonomic functions, such as regulation of blood pressure, and inability to breathe or cough. The long-term disability from SCI results not only from the initial loss of function, but also from the complications that accumulate. Up to 30% of individuals with SCI are hospitalized every year for medical complications. Patients who maintain their body in the best condition for nervous system repair will benefit most from future therapeutic strategies.

SPINAL CORD ORGANIZATION

Unlike the brain, the spinal cord has its white matter (nerve axons) on the outside and gray matter (cell bodies) on the inside (Figure 55-1). The gray matter houses neurons that project to the periphery at that level, to control movement and receive sensory signals. The white matter carries axonal connections to and from the brain, and half of all those axons are myelinated. Most traumatic SCIs also injure the incoming and outgoing (or afferent and efferent) peripheral nerves at the injury level. In most SCIs, however, the caudal cord remains intact, beginning several segments below the injury level. Circuitry in those segments can produce reflexes by activating a ventral motor neuron (MN) to produce muscle contraction when it receives a sensory stimulus from the periphery of the body (Figure 55-1).

The distal spinal cord also contains groups of nerve cells that generate the patterns of activity needed for walking and running. Because the upper spinal cord plays only a limited role in controlling these pattern generators, people with injury to the cervical spine can walk or ride a stationary bicycle if their muscles are stimulated appropriately.

INJURY

Traumatic injury occurs when broken fragments of bone and ligament impinge on the soft spinal cord, which is no wider than the thumb. The cord responds by swelling and the swelling compounds the initial injury by reducing venous blood flow, causing a secondary venous infarct in the central part of the cord. This causes the initial injury site to rapidly enlarge into a hole in the middle of the spinal cord (Figure 55-2). Because a donut-like rim of viable tissue usually remains at the level of injury, SCI preferentially affects gray matter.

A second wave of delayed cell death occurs during the weeks after an SCI. It removes mostly oligodendrocytes from adjacent white matter tracts. Since each oligodendrocyte myelinates 10 to 40 different axons, loss of one oligodendrocyte leads to exponential loss of myelin and function.

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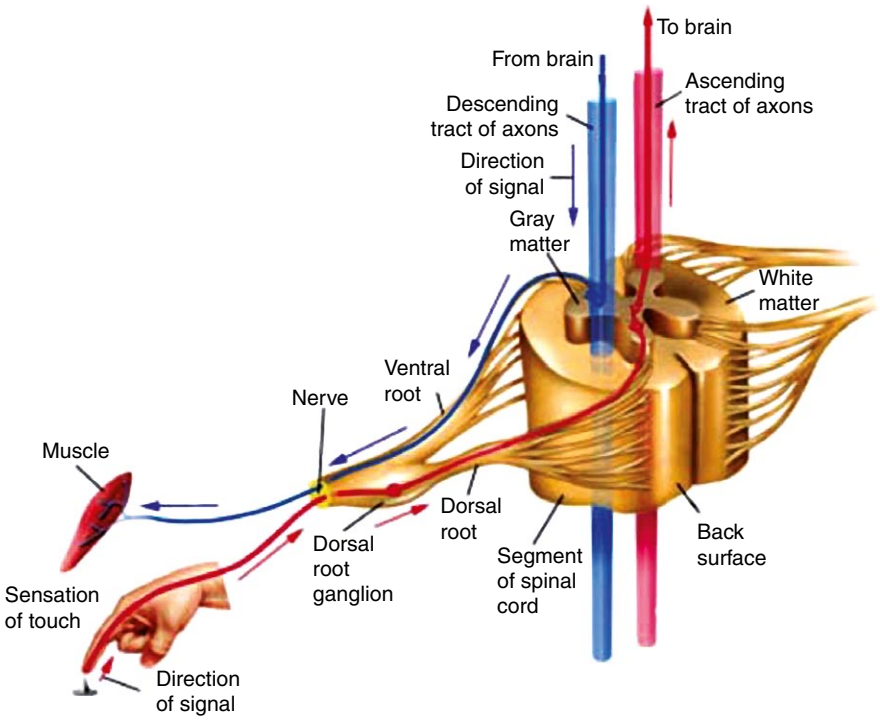


FIGURE 55-1 Organization of the spinal cord. A segment of cord reveals the butterfly-shaped gray matter at the core and a ring of white matter. The main components of the gray matter are neuronal cell bodies and glial cells and blood vessels. The outer white matter also contains astrocytes and blood vessels, but it consists mostly of axons and oligodendrocyte glial cells that wrap axons in white, insulated myelin. Axonal tracts that ascend in the cord convey sensory messages received from the body; the descending tracts carry motor commands to muscles. Reproduced with permission of Alexander and Turner Studio, FL, © 2002 Edmond Alexander.

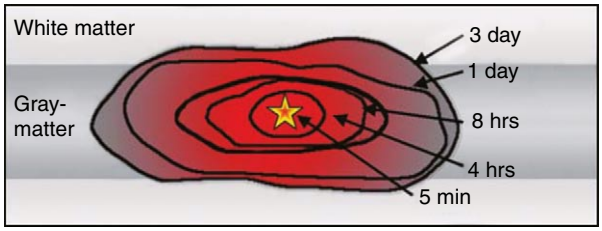


FIGURE 55-2 Evolution of primary to secondary injury in the spinal cord. Following the initial trauma, the injury rapidly enlarges as a consequence of secondary injury, particularly over the first 24 hours.

The problem does not stop with the secondary wave of cell death, however. An injured, underactive nervous system may be unable to adequately replace cells, particularly glial cells that normally turn over. Therefore, individuals with SCI may experience a slow, progressive loss of neurological function over long periods, in addition to complications from their initial injury.

SPONTANEOUS REGENERATION

A growing body of evidence indicates that the capacity for spontaneous regeneration may be much greater than previously perceived. Data obtained since the 1960s has demonstrated that new cells are added to the nervous system continually; these cells include neurons in at least two brain regions, the hippocampus and olfactory bulb. Moreover, glial cells are frequently born and are capable of regeneration.

Nonetheless, our ability to maximize spontaneous regeneration is limited. The recent detection of endogenous stem cells within the spinal cord raises hope that such cells can be harnessed to repair the damaged spinal system. Although the birth of new neurons from these progenitors has never been demonstrated within the spinal cord, new glial cells, including oligodendrocytes and astrocytes, are continually being added. In fact, injury stimulates cell birth. Our laboratory recently showed that nearly two million cells are born in the spinal cord each day, although most eventually die. Collectively, these data suggest that cell turnover does occur in the nervous system, albeit much more slowly than in other organs of the body. Moreover, we recently showed that patterned neural activity can stimulate cell birth, which suggests that behavioral modification could be important for maximizing cord regeneration and functional recovery.

LIMITATIONS AND APPROACHES TO REPAIR AND REDEFINING GOALS

Although some limited spontaneous regeneration is known to occur, dramatic self-repair of the nervous system does not take place. A growing body of evidence suggests that factors in the nervous system actively inhibit regeneration. Such factors include inhibitory proteins in the spinal cord, which guide regrowing connections, and scar tissue, which contains chondroitin sulfate and proteoglycans.

Figure 55-3 and Figure 55-15 outline the general strategies for repairing the damaged spinal cord. Complete cure of nervous system injury is not practical or required. Partial repair translates into proportionally greater recovery of function. For example, only about 10% of the functional

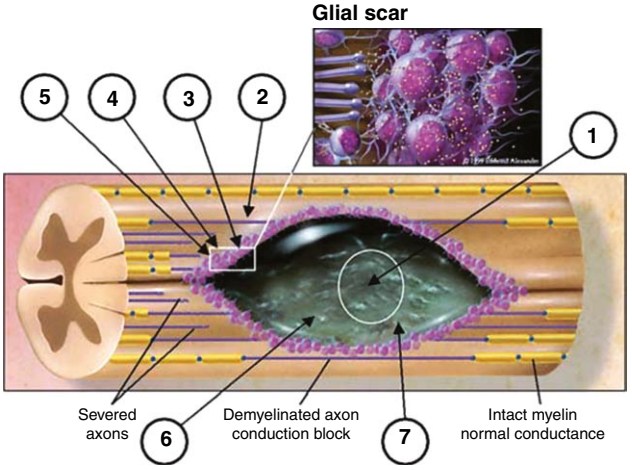


FIGURE 55-3 Common strategies toward regeneration of the damaged spinal cord. (A) Prevention of progression of secondary injury: necrotic and apoptotic cell death would be prevented by antiexcitotoxic drugs and antiapoptotic treatments. (B) Compensation for demyelination: chemicals that prevent conduction block in demyelinated areas, and agents that encourage surviving oligodendrocytes to remyelinate axons, would be provided. Lost oligodendrocytes would be replenished. (C) Removal of inhibition: agents that block the actions of natural inhibitors of regeneration or drugs that down-regulate expression of inhibitory proteins would be provided. (D) Promotion of axonal regeneration: growth factors that promote regeneration (sprouting) of new axons would be provided. (E) Direction of axons to proper targets: guidance molecules would be provided, or their expression would be increased in host cells. (F) Creation of bridges: bridges would be implanted into the cyst, which would provide directional scaffolding that encourages axon growth. (G) Replacement of lost cells: cells capable of generation of all cell types (progenitor cells or ES cells) would be implanted. Substances that induce undifferentiated cells to replace dead cells would be provided. Also, transplanted cells to deliver regenerative molecules would be used. Reproduced with permission of Alexander and Turner Studio, FL, © 2002 Edmond Alexander.

connections are required to support locomotion in cats, and humans missing more than half the gray matter in the cervical spinal cord can walk fairly normally.

One strategy for spinal cord repair is to transplant stem cells or other biomaterials to fill the cyst that forms at the eye of the injury. This cyst acts as a physical barrier to the growth of anatomically-intact axons in the surrounding donut of white matter. As well as filling in the cyst, it may be necessary to repair axons that no longer function properly because they have lost their myelin or have been inappropriately myelinated. Several approaches have been used to overcome this problem, which can manifest itself as a segmental conduction block. For example, potassium channels on dysfunctional axons can be blocked pharmacologically, and pre-existing or transplanted oligodendrocytes and their progenitors can be encouraged to make new myelin.

Another strategy is to remove or mask the effects of proteins in the glial scar around the cyst that actively inhibit the regrowth of new connections. Antibodies that block the inhibitory effects of these proteins promote sprouting. Moreover, certain growth factors promote the self-repair of physically broken connections. It is important to understand the feasibility of various repair strategies, and to redefine appropriate goals of repair. If we rank the preceding strategies in terms of likely success, it becomes clear that it will be difficult to persuade axons to regrow across a lesion, extend down the spinal cord, and make connections with the appropriate target cells. Remyelination seems more feasible, because it occurs continually at a steady rate in the damaged spinal cord. It is important to investigate all the possible strategies, however, because some will materialize sooner and some later. Most importantly, we must understand that multiple strategies delivered over time will be more important than the elusive magic bullet.

When defining appropriate goals for therapy, improving patients' quality of life must rank first. To this end, strategies that limit complications are important. Moreover, individuals with SCI often value small gains in function more highly than larger gains, such as walking. Thus, the top goal for most patients is recovery of bowel, bladder, or sexual function. Distant second includes recovery of respiratory function and the use of a single hand.

SPINAL CORD DEVELOPMENT

To understand spinal cord regeneration, it is necessary to understand spinal cord development. In humans, oligodendrocytes are found in cultures of fetal spinal cord at 7 and 12 weeks of gestation. Myelination begins at 10 to 11 weeks of gestation, and continues throughout the second year of life. Signals derived from axons regulate the growth of progenitor cells and the survival of oligodendrocytes. Other locally-synthesized growth factors appear to control the balance between oligodendrocyte precursor (OP) proliferation and OP differentiation.

Many neuronal phenotypes arise from various progenitor pools during CNS development, but most of the pathways are poorly understood. One of the best described is the generation of spinal motorneurons (MN), which involves several steps. Through bone morphogenetic protein, fibroblast growth factor (FGF), and Wnt signaling, ectodermal cells obtain a rostral neural character. In response to caudalizing signals, such as retinoic acid (RA), these progenitors acquire a spinal positional identity. Through the ventralizing action of Sonic hedgehog (Shh), spinal progenitor cells gain their MN phenotype.

These findings raise the question of whether embryonic stem (ES) cells can be shunted along specific pathways to produce specific neural cell populations for CNS repair. Wichterle and colleagues (2002) demonstrated that signaling factors operating along the rostrocaudal and dorsoventral axes of the neural tube to specify MN fate *in vivo* could be harnessed *in vitro* to direct the differentiation of mouse ES cells into functional MNs.

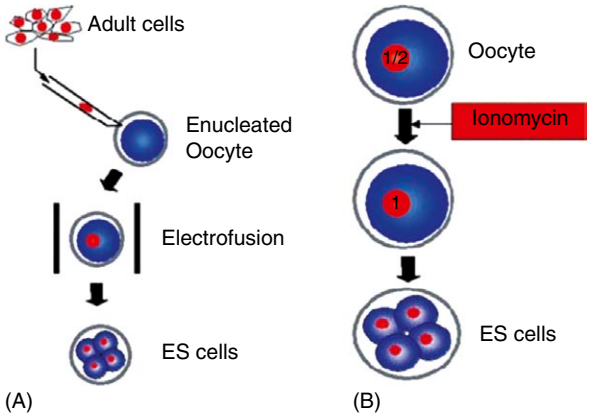


FIGURE 55-4 ES cells can be made by *in vitro* fertilization and two additional methods. (A) Somatic nuclear transfer: donor cells are placed under the zona pellucida into the perivitelline space of enucleated oocytes. The cell nucleus is introduced into the cytoplast by electrofusion, which also activates the oocyte. It can then be grown to the blastocyst stage to harvest the ES cells. (B) Parthenogenic activation: for activation, oocytes are briefly exposed to a calcium ionophore, such as ionomycin. The resulting cell can mature for ES cell harvest.

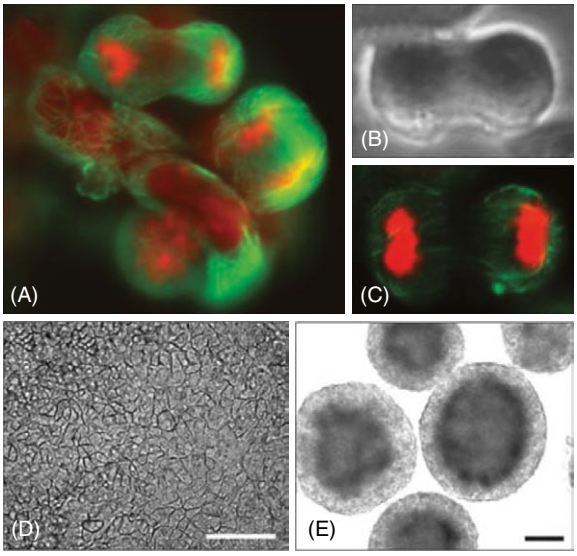


FIGURE 55-5 Undifferentiated mouse and human ES cells dividing in a culture dish. (A, C) Immunofluorescence images of mouse ES cells demonstrate anti-β-tubulin and anti-DNA (Hoechst). The phase image of (C) the identical field is shown in panel B. Panel D illustrates undifferentiated human ES cells. EBs derived from human ES cells are shown in panel E. Scale bar for panels D–E = 100 μm.

EMBRYONIC STEM CELLS

Embryonic stem cells have unique features that are important for spinal cord repair. They represent every cell type in the body, are the earliest stem cells capable of replicating indefinitely without aging, and their DNA can be modified easily, even in single cells. They also fulfill two criteria essential for nervous system repair: transplantable cells should be cells that normally belong in the spinal cord; and transplantable cells should contain the recipient's own DNA to avoid the need for immunosuppression, which cannot be used in spinal cord patients because of the increased incidence of infections.

Several methods are available for obtaining ES cells. The most common is *in vitro* fertilization, which requires a fertilized egg, and therefore produces cells that differ genetically from the host. A second strategy involves somatic nuclear transfer, which takes a nucleus from a somatic cell, such as skin, and transfers it to an enucleated fertilized egg (Figure 55-4A). The subsequent ES cells therefore contain only the genetic material of the recipient. Another possibility for women is parthenogenesis, which tricks an egg into thinking it is fertilized, allowing it to begin duplicating its DNA (Figure 55-4B). This process is unable to create a viable embryo (because factors derived from sperm are necessary for pre-implantation), but it can produce normal cells.

A novel tool for obtaining stems cells was the discovery of induced pluripotent stem cells (iPS cells). Now, for the first time, by expressing four genes, scientists can convert adult cells, such as skin and other cells, to pluripotent stem cells. This tool will most likely revolutionize the field of stem cell repair strategies, not only in the CNS, in the years to come.

Many studies of spinal cord repair have involved differentiated cells. The earliest studies used peripheral nerve grafts to demonstrate that the nervous system has the capacity to regenerate, but that the environment in the CNS is not permissive. Substantial progress has been made with the transplantation of peripheral nervous system cells and non-nervous system cells. In general, fetal sources of cells have been the most successful, because derivatives of postnatal and adult cells are less able to withstand neural transplantation. One recent exception has been neural stem cells derived from the adult CNS.

EMBRYONIC STEM CELLS AND THE NEURAL LINEAGE

Several protocols are available for converting ES cells into neural lineage cells. However, protocols for mouse and human cells differ (Figure 55-5). Differentiating murine ES cells traditionally begins with floating spheres called embryoid bodies (EBs). These bodies are akin to the neural spheres of stem cells obtained from the adult CNS. Retinoic acid is a key induction agent for producing neural progenitors from mouse ES cells. Neural cells resembling anatomically normal neurons, astrocytes, and oligodendrocytes from the CNS can easily be derived from mouse ES cells using these protocols (Figure 55-6).

EMBRONIC STEM CELL TRANSPLANTATION

Use of ES cells for neural transplantation is in its infancy, and only a limited amount of work has been completed

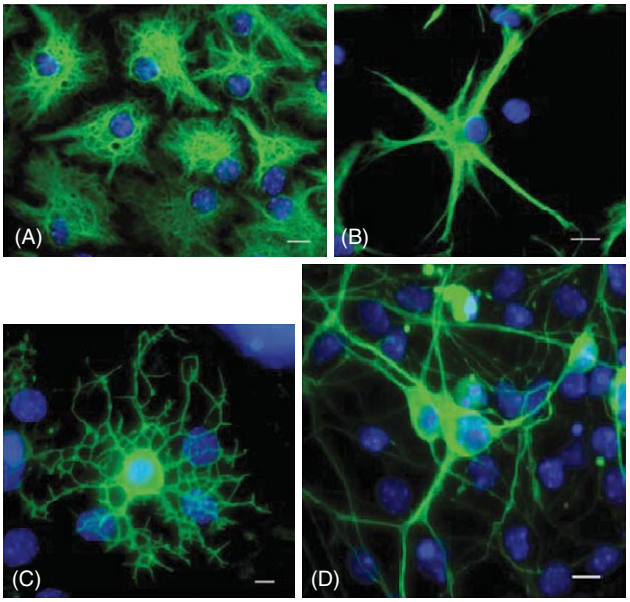


FIGURE 55-6 ES cells differentiate into the principal types of neural cells. (A) Type I; and (B) type II astrocytes (anti-GFAP); (C) oligodendrocytes (anti-O1); and (D) neurons (anti-β-tubulin). Scale bar = 10 μm. Reproduced with the permission of Becker *et al.*, 2003.

with the spinal cord. Overall, the studies demonstrate that ES cells have a remarkable ability to integrate into the injured region of the cord and differentiate appropriately.

ES cells can participate in the normal development of spinal cord cells, including MNs. It has been demonstrated that developmentally relevant signaling factors can induce mouse ES cells to differentiate into spinal progenitor cells, and then to MNs, through the normal developmental pathway (Figure 55-7). Thus, the signals that promote the differentiation of neural stem cells *in vivo* are effective when applied to ES cells. MNs derived from ES cells can populate the embryonic spinal cord, extend axons, and form synapses with target muscles (Figure 55-8). Therefore, they not only participate in normal development, but also grow appropriately when transplanted into the embryonic spinal cord, targeting muscle. Thus, inductive signals in normal pathways of neurogenesis can direct ES cells to form specific classes of CNS neurons.

Brustle, Duncan, and McKay demonstrated that ES cells transplanted into the brain and the spinal cord of normal adult animals can differentiate into oligodendrocytes that can myelinate axons. They generated OPs efficiently by supplementing cultures of ES cells with FGF and epidermal growth factor and later including PDGF. To investigate whether these oligodendrocytes could myelinate *in vivo*, cells grown in the presence of bFGF and PDGF were injected into the spinal cord of one-week-old myelin-deficient rats. Two weeks after transplantation, numerous myelin sheaths were detected in six of the nine affected rats; the original 100 000 cells had migrated widely, and made myelin with appropriate ultrastructure.

This group also transplanted ES cell-derived precursors into the cerebral ventricles of developing rodents (embryonic day (E) 17). Three weeks later, proteolipid protein-positive myelin sheaths were evident in a variety of brain regions. The cells' exogenous origin was confirmed by *in situ* hybridization with a probe to mouse satellite DNA. There was no observable evidence of abnormal cellular differentiation or tumor formation.

In the same year, McDonald, Gottlieb, and Choi demonstrated for the first time that ES cells that had been induced to become neural cell precursors could be successfully transplanted into the injured spinal cord. Examination of the spinal cord nine days after one million precursor cells were transplanted into a cyst caused by contusion injury showed that the cells had survived, grafted, migrated long distances, and differentiated into the three principal neural cell types: neurons, astrocytes, and oligodendrocytes (Figures 55-9 and 55-10). Transplantation was associated with a significant and sizable improvement in function. Their study was the first demonstration that transplanted embryonic precursors can successfully repair the damaged adult nervous system. This is an important finding, given that conditions in the damaged adult nervous system are much less favorable than those in the neonatal spinal cord. Subsequently, Liu and colleagues (2000) demonstrated that stem cell-derived precursors transplanted into the injured adult nervous system can achieve substantial remyelination with appropriate anatomical characteristics (Figure 55-11). Furthermore, they showed that ES cell-derived oligodendrocytes functioned normally, myelinating multiple axons in culture, just as they do in the normal nervous system (Figure 55-12). Using patch clamp analysis, Huettner and McDonald later demonstrated that the physiological characteristics of ES cell-derived oligodendrocytes are similar to those of oligodendrocytes taken from the adult spinal cord (Figures 55-12C and 55-13). ES cell-derived oligodendrocytes represent the entire oligodendrocyte lineage, from early OPs to mature, myelinating oligodendrocytes.

In culture, ES cell-derived neurons rapidly differentiate, and spontaneously create neural circuits with anatomical (Figure 55-14) and physiological evidence of excitatory and inhibitory synapses. Substantial neural differentiation is also evident *in vivo* in the model of contusion injury, where neurons show extensive axonal outgrowth with presumptive morphological evidence of synapse formation. Moreover, immunological evidence of cholinergic, serotonergic, GABAergic, glycinergic, and glutamatergic neurons has been obtained *in vitro* and *in vivo*.

Implantation, survival, and migration of transplanted ES cell-derived precursors in the damaged spinal cord have been verified by using magnetic resonance imaging to track oligodendrocytes prelabeled with paramagnetic agents, as well as real-time polymerase chain reaction. In both cases, migration up to 1 cm from the transplantation site was evident.

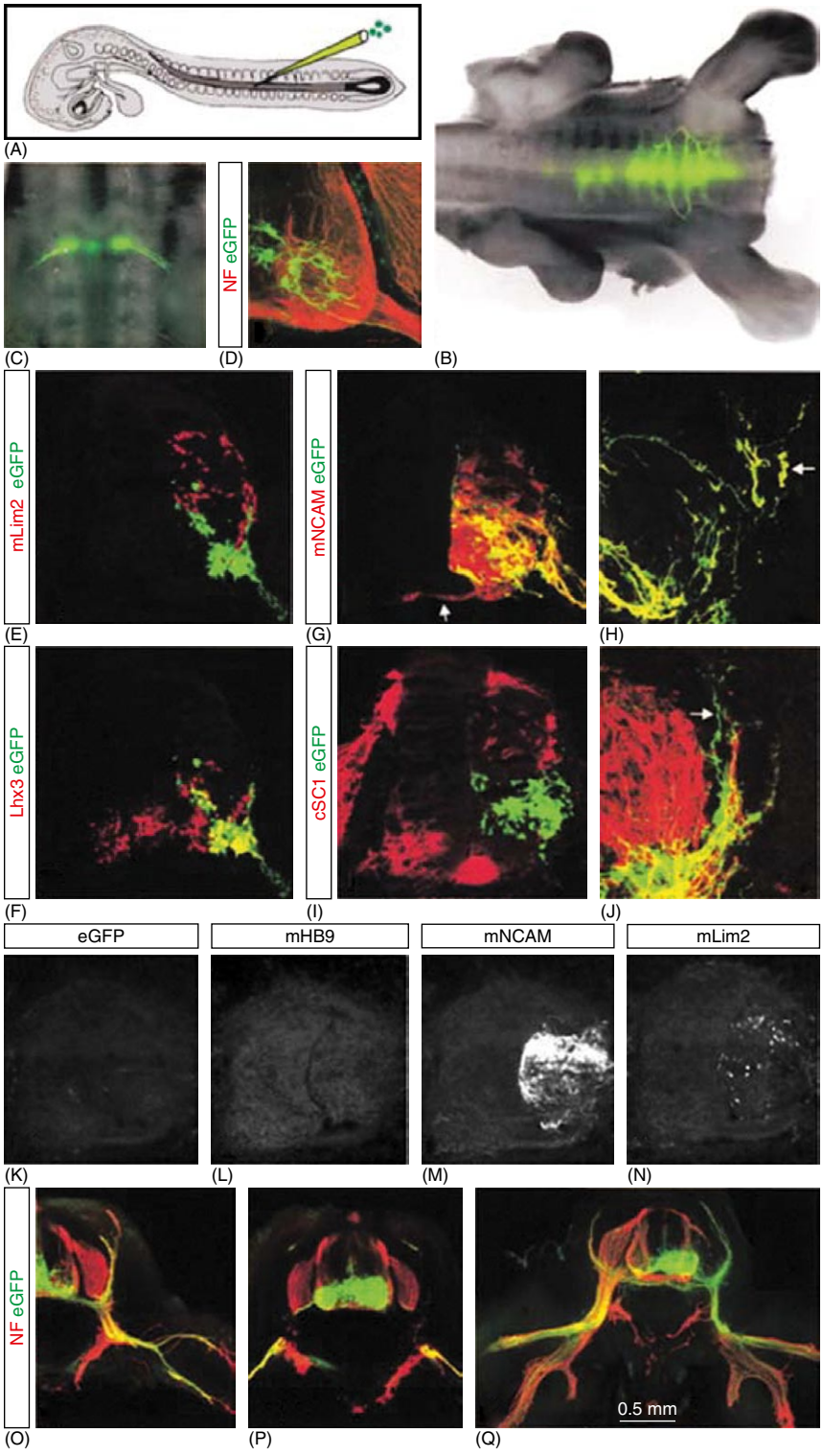


FIGURE 55-7 Embryonic transplantation of MNs derived from mouse ES cells. Integration of transplanted ES cell-derived MNs into the spinal cord *in vivo*. (A) Implantation of HBG3 ES cell-derived MN-enriched EBs into stage 15–17 chick spinal cord. (B) Bright-field–fluorescence image showing eGFP MNs in thoracic and lumbar spinal cord, assayed at stage 27 (ventral view). (C, D) Location of FACS, ES cell-derived, eGFP MNs in thoracic spinal cord, assayed at stage 27. (D) eGFP MNs are clustered in the ventral spinal cord. (E–J) Transverse sections through stage 27 chick spinal cord at rostral cervical levels after transplantation of MN-enriched EBs. (E) MNs are concentrated in the ventral spinal cord and are segregated from transplanted interneurons, labeled by a mouse-specific Lim2 antibody. (F) Many ES cell-derived MNs coexpress eGFP and Lhx3. (G) ES cell-derived MNs; and (H) axons (arrow) are labeled by (I, J) rodent-specific anti-NCAM antibody, but do not express the chick MN marker protein SC1. (G, H) eGFP, NCAM axons cross the floor plate but do not project out of the spinal cord (arrows). (K–N) Transverse sections of thoracic spinal cord at stage 27, after grafting EBs grown with RA (2μM) and anti-Hh antibody (5E1, 30μg/ml). No mouse-derived MNs were detected either: (K) by eGFP; or (L) by a mouse-specific anti-HB9 antibody. In contrast, many mouse-derived: (M) NCAM; and (N) Lim2 interneurons are present. (O–Q) Transverse sections through stage 27 spinal cord at: (O, P) thoracic; and (Q) lumbar levels after grafting MN-enriched EBs. eGFP MNs are concentrated in the ventral spinal cord. Ectopic eGFP MNs are located within the lumen of the spinal cord. eGFP axons exit the spinal cord primarily through the ventral root and project along nerve branches that supply: (O–Q) axial; (O, P) body wall; and (Q) dorsal and ventral limb muscles. The pathway of axons is detected by neurofilament (NF) expression. eGFP axons are not detected in motor nerves that project to sympathetic neuronal targets. Reproduced with the permission of Wichterle *et al.*, 2002.

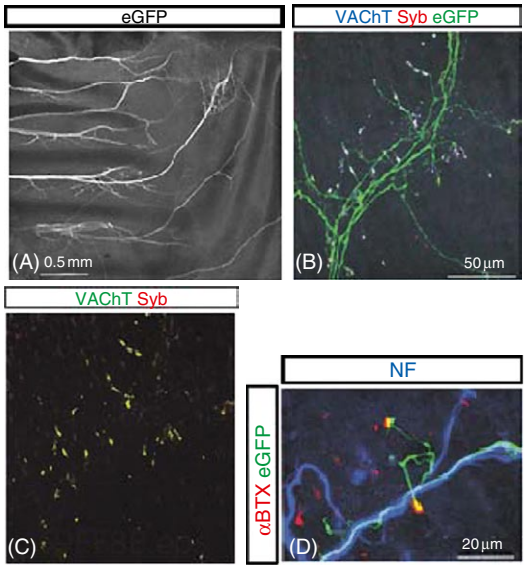


FIGURE 55-8 Anatomic integration of MNs derived from transplanted mouse ES cells. Synaptic differentiation of ES cell-derived MNs *in vivo*. (A) Whole-mount preparation of stage 35 chick embryonic rib cage. ES cell-derived eGFP motor axons contact intercostal muscles. (B, C) Coexpression of synaptobrevin (Syb) and vesicular ACh transporter (VAChT) in the terminals of eGFP motor axons at sites of nerve contact with muscle. The anti-Syb and VAChT antibodies recognize mouse but not chick proteins. (D) NF and eGFP expression in motor axons that supply intercostal muscles. eGFP axons lack NF expression. The terminals of eGFP axons coincide with clusters of ACh receptors, defined by α-bungarotoxin (αBTX) labeling. (E) Coincidence of synaptotgamin (Syn) expression in eGFP motor axon terminals and αBTX labeling. (F) Coincidence of Syb expression in eGFP motor axon terminals and αBTX labeling. Reproduced with the permission of Wichterle *et al.*, 2002.

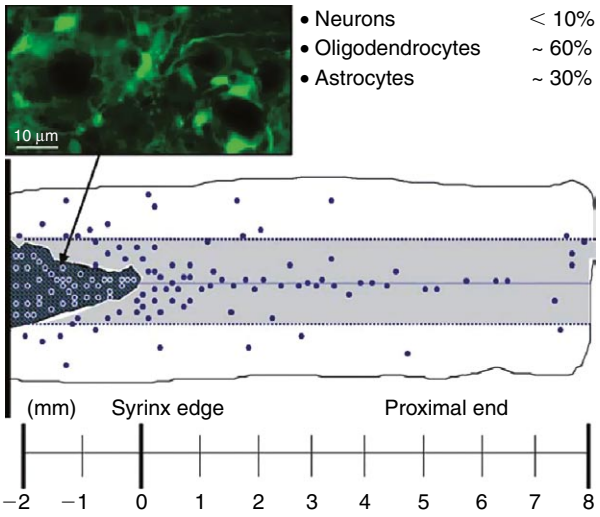


FIGURE 55-9 In the contusion-injured spinal cord of the rat, ES cell-derived neural precursors survive, migrate, and differentiate following transplantation. Schematic demonstrates the relative distribution of ES cell-derived cells two weeks after transplantation into the forming central cavity, which was done nine days after spinal contusion injury. The cavity is partially filled, and cells migrate long distances. Most distant cells are identified as oligodendrocytes, and astrocytes and neurons remain restricted to the site of transplantation. The inset shows GFP-expressing ES cell-derived neural cells.

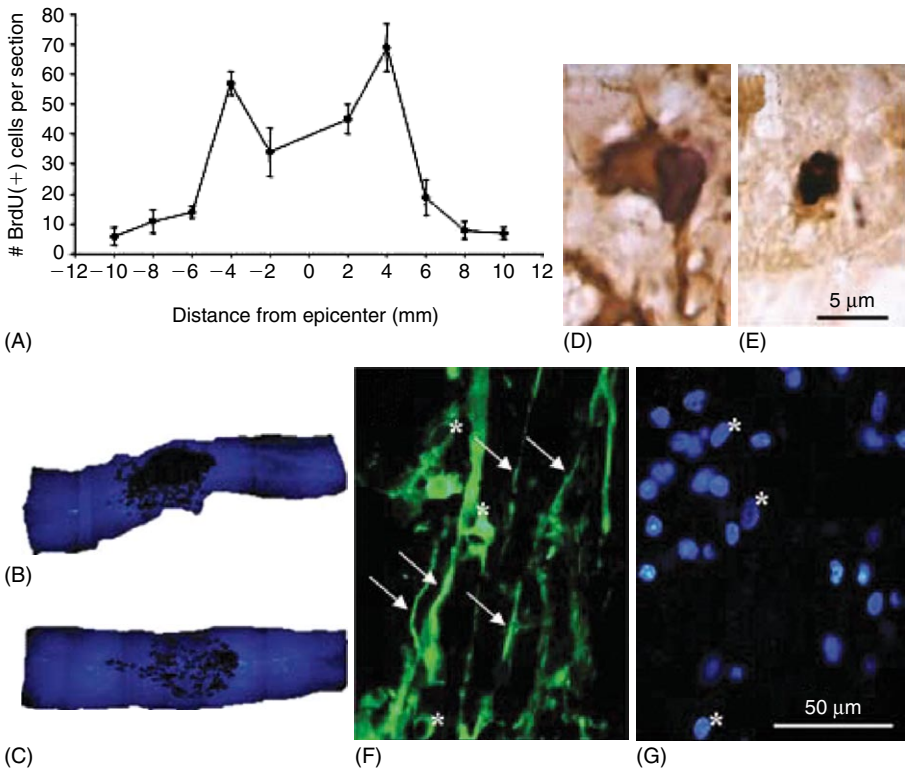


FIGURE 55-10 BrdU-labeled ES cell-derived cells two weeks after transplantation. Mean ± SEM BrdU-labeled nuclei per 1 mm segment in longitudinal sections. (A) Hoechst 33342-labeled sections 42 days after injury, transplanted with: (B) vehicle; or (C) ES cells nine days after injury. (D) BrdU cell co-labeled with GFAP. (E) BrdU-labeled cell co-labeled with APC CC-1. (F) The mouse-specific marker EMA indicates processes (arrows) emanating from ES cells. (G) Corresponding nuclei are marked by asterisks. Modified from McDonald *et al*. with permission.

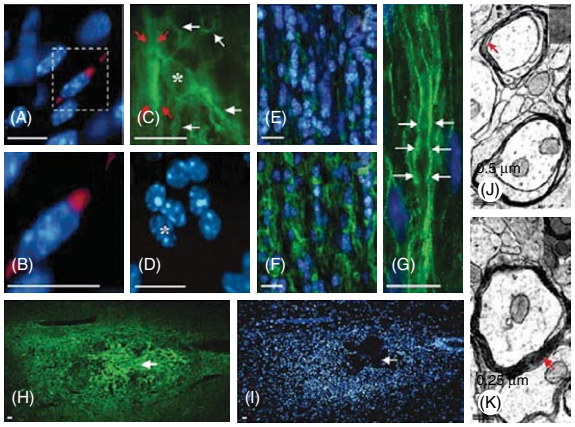


FIGURE 55-11 Cells derived from ES oligospheres can migrate and myelinate axons when transplanted into dysmyelinated spinal cords of adult *Shiverer* mice. Such mice lack the gene for myelin basic protein (MBP). Transplanted cells were identified by Cell Tracker Orange (CTO), epifluorescence, or immunoreactivity for MBP. (A, B) CTO-labeled cells aligned with native intrafascicular oligodendrocytes in white matter. (C, D) An ES cell-derived (MBP) oligodendrocyte (asterisk) with longitudinally oriented processes (white arrows) is shown. (C) Arrows mark probable myelination around an adjacent axon. (E) Little MBP immunoreactivity is seen in white matter in a longitudinal section of spinal cord from a mouse that received a sham transplantation. (F) A gradient of MBP immunoreactivity centers on the site of ES cell transplantation. (G) High magnification of intrafascicular oligodendrocyte nuclei and MBP immunoreactivity, two indications of axonal myelination (white arrows), in white matter from a mouse transplanted with ES cells. (H) The spatial distribution of MBP immunoreactivity 1 month after ES cell transplantation is shown at low magnification, with (I) corresponding Hoechst 33342 counterstaining. White arrows indicate the center of the transplant site. (J) Transmission EM shows four loose wraps of myelin, the maximal number of layers typically seen around axons in control animals (arrow); and (K) nine or more compact wraps around axons from the transplanted area (arrow). *Shiverer* mutant mice lack a functional *MBP* gene required to form mature compact myelin; therefore, the presence of mature compact myelin is a standard for transplant oligodendrocyte-associated myelin. Scale bars = 10 µm (A–I) and 0.3 µm (J, K). Reproduced with the permission of McDonald *et al.*

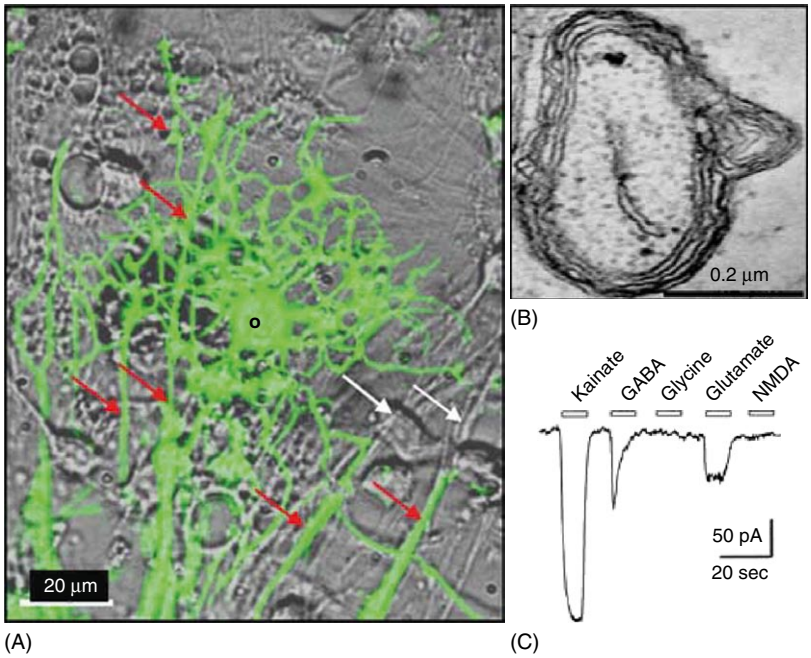


FIGURE 55-12 ES cells produced mature oligodendrocytes with normal anatomical features of myelination and physiological response to neurotransmitters. Reproduced in adapted form with permission from Liu *et al.*, 2000.

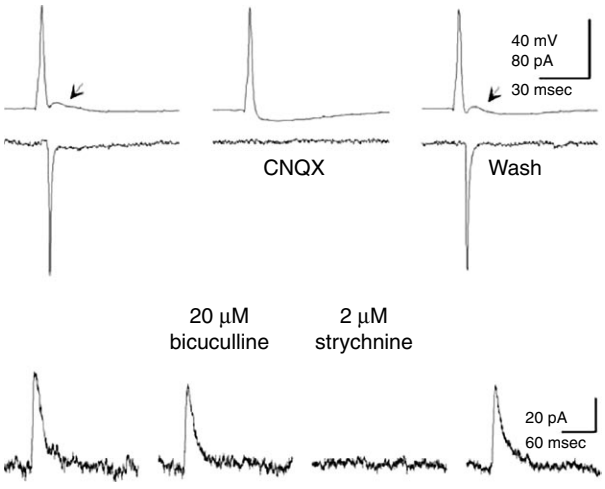


FIGURE 55-13 Excitatory and inhibitory synaptic transmission among neurons derived *in vitro* from mouse ES cells. Action potentials were evoked in the presynaptic neuron by current injection. Excitatory (top) postsynaptic currents, blocked by superfusion with the selective glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 µM), were recorded under a voltage clamp. Arrows point to an autaptic excitatory synaptic potential also blocked by CNQX. Inhibitory (bottom) synaptic currents were evoked in a different ES cell pair and tested sequentially with the antagonists bicuculline and strychnine. For this cell pair, only the postsynaptic currents are shown. Transmission was blocked by strychnine, indicating glycine was the transmitter. Other presynaptic cells evoked bicuculline-sensitive synaptic currents, indicating transmission mediated by GABA (not shown).

NOVEL APPROACHES TO CENTRAL NERVOUS SYSTEM REPAIR

Most neural transplantation studies, including those using ES cell precursors, have used cells to replace cells lost after injury. Given that we do not know which types of differentiated cells are required for repair, neural precursors may be ideal for this purpose, because environmental clues can decide their developmental fate. Moreover, ES cell-derived precursors can serve as bridges to support axonal regrowth.

In an attempt to overcome the constraints of the injured nervous system, ES cells are often genetically altered so that they will deliver growth molecules, such as NT3, after transplantation, and they are particularly well-suited to this task. McDonald and Silver recently adopted a novel approach to overcoming restraints by showing that early ES cell-derived progenitors can phagocytize key inhibitory molecules in glial scar tissue. These cells, therefore, created an inhibitor-free bridge over which axons could rapidly sprout from the transplant into normal cord. By nine days after transplantation, axons from the graft had grown up to 1 cm—a rate of more than 1 mm per day! This rate is similar to that seen in the normal embryo.

Another novel approach is to use stem cell transplantation to limit the secondary injury that occurs after nervous system injury. For example, we recently demonstrated that transplanting ES cells can limit the delayed death of neurons and oligodendrocytes. Since most transplantation studies are completed at the time of injury, it is possible that many of their results may be attributable to this neuroprotective role. It seems clear that even genetically-unmodified ES cells release large quantities of growth factors.

Although replacing lost neurons is difficult, it might be possible to use neurons to create bridging circuits across the injury site. Descending axons could synapse onto ES cell-derived neurons that subsequently synapse onto key pattern generators in the lower spinal cord. A more global delivery of neurotransmitters using synaptic or nonsynaptic mechanisms might enhance functions such as locomotion. Previous work by others has demonstrated that release of noradrenergic and serotonergic neurotransmitters can stimulate and enhance the central pattern generator for locomotion. More recent work indicates that release of the neurotrophins BDNF and NT3 can perform this function.

Because ES cells are embryonic in nature, their progenitors may also be able to reprogram the adult CNS so that damage can be repaired. ES cells are also unique, in that they have the potential to replace cells derived from multiple embryonic germ layers. In the CNS, it will be important to replace lost non-neural cells, as well as neural cells, to regain normal CNS function, such as neovascularization. The growing link between neovascularization and neurogenesis strengthens this approach. We recently found that ES cells in transplanted 4/4 EBs differentiate into both neural cells and vascular endothelial cells.

Finally, remyelination (Figure 55-15) is one of the most pragmatic approaches to restoring function to the damaged spinal cord. Because many potentially functional connections remain in the outer donut of surviving tissue, appropriate remyelination could substantially improve function. One must consider, however, that dysmyelination rather than demyelination is the biggest problem in the damaged nervous system.

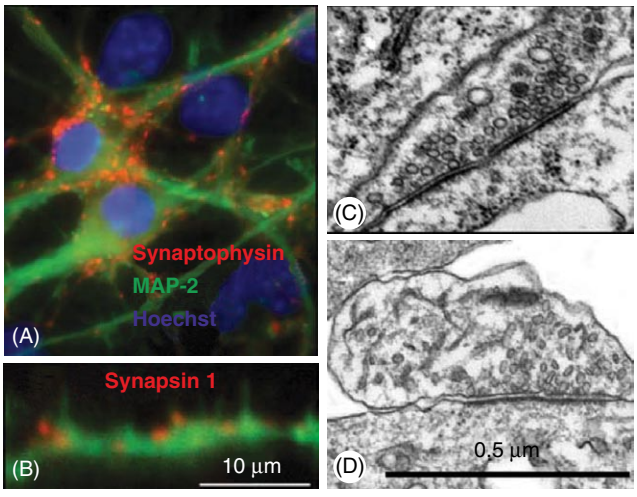


FIGURE 55-14 ES cells differentiate into neurons that spontaneously create neural circuits. (A, B) Presumptive presynaptic sites oppose dendrites. (C, D) Ultrastructural characteristics of synaptic profiles *in vitro*.

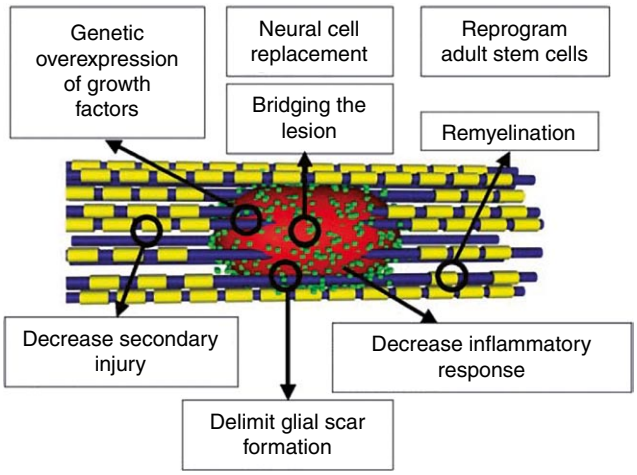


FIGURE 55-15 Novel approaches to spinal cord repair using ES cells. Remyelination is one of the most pragmatic approaches to restoring function to the damaged spinal cord. ES cell-derived precursors can serve as bridges to support axonal regrowth. They can phagocytize key inhibitory molecules in glial scar tissue. These cells therefore created an inhibitor-free bridge over which axons could rapidly sprout from the transplant into normal cord. Newly generated neurons can be used to create bridging circuits across the injury site. ES cell transplantation can limit the secondary injury. To try to overcome the constraints of the injured nervous system, ES cells are often genetically altered so they will deliver growth molecules. Because ES cells are embryonic in nature, their progenitors may also be able to reprogram the adult CNS to optimize spontaneous host regeneration. Replacement of nonneural cells as well as neural cells will be important to regain normal CNS function, such as neovascularization.

TOWARD HUMAN TRIALS

Some of the animal studies described previously have prompted early human safety trials. Most have focused on Parkinson's disease. Phase 1 human trials for repairing the spinal cord have included transplantation of porcine-derived stem cells for the purpose of remyelination (USA), as well as allogeneic transplantation of olfactory ensheathing glia (Portugal, Germany). Dr Wise Young, utilizing the China Spinal Cord Injury Network, is comparing the use of human umbilical cord blood mononuclear cell (UCBM) transplants with and without Lithium in a Phase 3 trial. A United States Spinal Cord Injury Network is in preparation. It is important to note that such trials are paving the way for further uses of ES cells. With the recent advent of human ES cells, human transplantation appears more feasible, although somatic transplantation will be required to ensure a genetic match. Such manipulation of cells raises many regulatory concerns that must be addressed before clinical trials can move forward. The recent demonstration of *in vitro* oocyte generation from mouse ES cells raises the possibility that ethically acceptable procedures for human nuclear transfer may soon be available.

SUMMARY

Studies of neurotransplantation for repairing the damaged nervous system are making good progress. Early animal studies showed that mouse ES cells can: replace neurons, astrocytes, and oligodendrocytes; instigate appropriate remyelination; and even improve locomotion. As is the case for all transplantation studies, however, the mechanisms underlying functional improvement remain unclear. Nevertheless, ES cells offer a novel approach to deciphering these mechanisms.

Although it is impossible to predict the future of the field, it is clear that murine and human ES cells are tools that will revolutionize neurobiology and neural transplantation, by providing the unprecedented ability to selectively deliver key regulatory factors. Also, ES cells promise to be one of the greatest therapies for chronic nervous system disorders. Only the future will reveal their full potential for treating human disease and disability, but it is possible to say with confidence that ES cells will have a major effect on repairing the human CNS within our lifetimes.

KEY WORDS

Embryoid body A collection of hundreds of cells that resemble the early embryo, which is produced by growing ES cells in the presence of retinoic acid. These bodies contain neural tube-like structures, and have been successfully used to repair the damaged spinal cords of rats.

Parthenogenesis The process of tricking a nonfertilized egg into duplication of DNA to produce an all-female-derived embryonic stem cell.

Remyelination The process of repair that is predicted to most likely lead to return of limited function, without requiring new circuits to form. Oligodendrocytes are the glial cells that wrap (mylinate) neuronal axons. New data suggests that remyelination is one of the processes that can occur spontaneously after injury.

Somatic nuclear transfer A method that involves transferring a nucleus from a somatic (e.g., skin cell) to an enucleated fertilized egg. This produces an embryonic stem cell clone of the somatic cell DNA.

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Chapter 56

Use of Embryonic Stem Cells to Treat Heart Disease

Michael Rubart and Loren J. Field

INTRODUCTION

It is now well-established that cardiomyocytes can be stably transplanted into normal or injured adult hearts. Moreover, transplanted donor cells can form a functional syncytium with the host myocardium. It is also well-established that embryonic stem (ES) cells can differentiate into functional cardiomyocytes *in vitro*, and that these ES-derived cardiomyocytes form stable grafts when transplanted into the myocardium. ES cells might thus represent a suitable source of donor cardiomyocytes for cell transplantation therapies aimed at restoring lost myocardial mass in diseased hearts. In this chapter, an overview of the use of cell transplantation as a paradigm for the treatment of diseased hearts is provided. This is followed by a discussion of ES-derived cardiomyocytes, and factors that enhance cardiomyogenic differentiation. Transplantation of ES-derived cardiomyocytes in preclinical studies, and challenges for their clinical implementation, are then considered.

CARDIOMYOCYTE TRANSPLANTATION AS A PARADIGM FOR THE TREATMENT OF DISEASED HEARTS

Cell transplantation has emerged as a potential therapeutic intervention to enhance angiogenesis, provide structural support, and perhaps even to restore lost myocardial mass in diseased hearts. To date, a number of donor cell types have been tested in preclinical studies for their ability to attain these goals, including skeletal myoblasts, fetal cardiomyocytes, angioblasts, vascular precursors, bone marrow stem cells, mesenchymal stem cells, ES-derived cardiomyocytes, and a variety of cardiac-resident stem cells with alleged cardiomyogenic activity. In many cases, cell transplantation was associated with improvement in cardiac function secondary to beneficial post-injury myocardial remodeling, and perhaps, to contractile activity of the donor cells. Clinical studies using skeletal myoblasts or crude mononuclear hematopoietic stem cell preparations from either bone marrow or peripheral blood have also been initiated. These initial clinical studies collectively established that cell transplantation in injured hearts is relatively safe, although the presence of life-threatening tachyarrhythmias in patients receiving skeletal myoblast transplants has necessitated defibrillator implantation. However, the impact of cell transplantation on cardiac function in patients has been mixed, with some studies demonstrating improvement in cardiac function and clinical outcome, and other studies demonstrating no effect.

Cardiomyocyte transplantation as a potential intervention to restore cardiac mass in diseased hearts was initially explored in the early 1990s. In these studies, single cell suspensions generated by enzymatic digestion of fetal hearts were transplanted directly into the ventricle of histocompatibility-matched or immune-suppressed recipient animals. The transplanted cardiomyocytes were identified by virtue of reporter transgene activity, or alternatively by the expression of an endogenous gene product that was absent in the recipient myocardium. The donor cells stably engrafted into the recipient hearts, underwent terminal differentiation, and expressed many of the molecular and morphological attributes typical of normal adult cardiomyocytes. Transmission electron microscopic analyses indicated that the transplanted donor cardiomyocytes formed mature intercalated discs with host cardiomyocytes comprised of fascia adherens, desmosomes, and gap junctions.

Subsequent studies demonstrated that transplanted cardiomyocytes formed a functional syncytium with the host myocardium. In these experiments, fetal cardiomyocytes from transgenic mice expressing enhanced green fluorescent protein (EGFP) were transplanted into the ventricle of histocompatibility-matched non-transgenic recipient animals. An image of a typical graft is shown in Figure 56-1A. To demonstrate the formation of a functional syncytium, transplanted hearts were harvested, and perfused on a Langendorff apparatus with rhod-2 (a calcium indicator dye that exhibits increased fluorescence with increased

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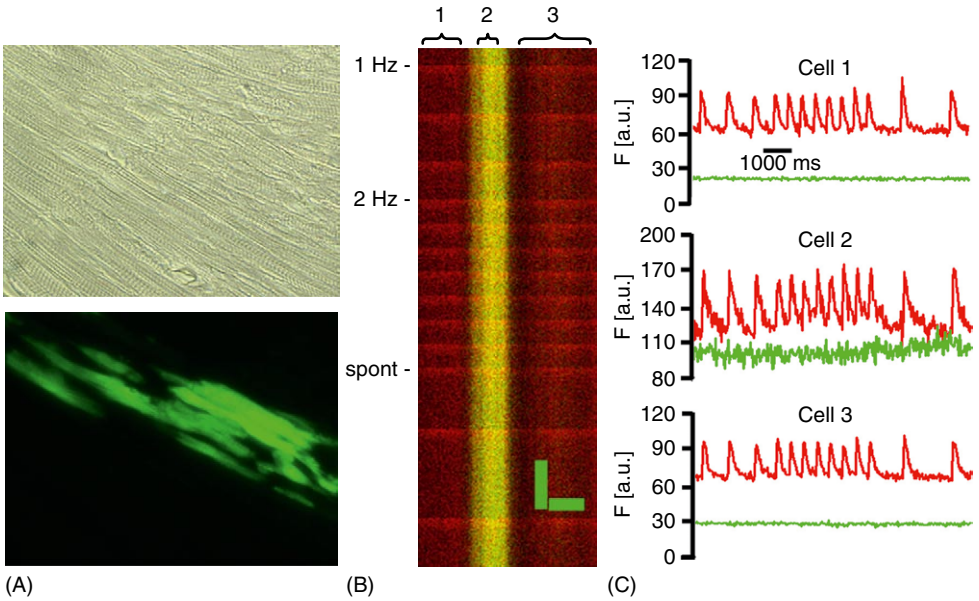


FIGURE 56-1 Functional coupling of donor cardiomyocytes with the host myocardium. (A) Brightfield (top) and epi-fluorescence (bottom) image of an area of a mouse left ventricle containing EGFP expressing donor cardiomyocytes and non-expressing host cardiomyocytes. The heart was harvested 27 days post-engraftment. (B) Simultaneous imaging of rhod-2 (red) and EGFP (green) fluorescence in a non-transgenic heart carrying an MHC-EGFP fetal cardiomyocyte graft. The image shows vertically-stacked line scans that traverse three juxtaposed cardiomyocytes. Cells 1 and 3 are host cardiomyocytes, whereas cell 1 is an EGFP-expressing donor cardiomyocyte. The heart was paced via point stimulation at a remote site at the rates indicated. (spont indicates spontaneous $[Ca^{2+}]_i$ transient.) Donor cells appear yellow, due to superimposition of red rhod-2 fluorescence and the green EGFP signal. Scale bars = 20 microns horizontally, 1000 microns vertically. Time runs from top to the bottom. (C) Spatially integrated traces of the changes in rhod-2 (red) and EGFP (green) fluorescence for the three cardiomyocytes in panel B. The signal across the entire cell was averaged, and plotted as a function of time. Note that both stimulated and spontaneous $[Ca^{2+}]_i$ transients occur simultaneously in donor and host cardiomyocytes, and exhibit similar kinetics.

intracellular calcium content) and cytochalasin D (an excitation-contraction uncoupler). The perfused hearts were then subjected to two-photon laser scanning microscopy under conditions which permitted simultaneous imaging of EGFP status (to distinguish the donor and host cardiomyocytes), and rhod-2 fluorescence (to monitor intracellular calcium transients, and, by inference, action potential propagation). Examination of intracellular calcium transients in neighboring donor and host cardiomyocytes revealed that they occurred simultaneously with, and were indistinguishable from, one another (Figure 56-1B and 56-1C). Thus, donor fetal cardiomyocytes were able to participate in a functional syncytium with the host heart.

EMBRYONIC STEM CELL-DERIVED CARDIOMYOCYTES, AND FACTORS WHICH ENHANCE CARDIOMYOGENIC DIFFERENTIATION

The studies described above indicated that cardiomyocyte transplantation might be a useful mechanism to restore lost function in diseased hearts. Since utilization of fetal donor cells is not practical, a surrogate source of donor cardiomyocytes would be required for clinical application. Embryonic stem (ES) cells are multipotent cells derived from pre-implantation embryos. ES cells have been amplified and maintained indefinitely in an undifferentiated state when grown in monolayer culture under appropriate conditions. When grown in suspension culture, ES cells formed multi-cellular aggregates known as embryoid bodies (EBs). Stochastic cell–cell interactions during EB formation mimicked normal developmental induction cues, and resulted in the random differentiation of ecto-, endo-, and meso-dermal cell lineages. Cardiomyogenic differentiation was frequently observed in differentiating EBs, and was easily recognized due to the presence of spontaneous contractile activity. EB formation has been successfully utilized to generate ES-derived cardiomyocytes from a large variety of species.

Cardiomyocytes derived from ES cells have been fairly well-characterized *in vitro*. ES-derived cardiomyocytes with molecular and electrophysiological characteristics of atrial, ventricular, or conduction system cells were readily identified. The temporal pattern of terminal differentiation of ES-derived cardiomyocytes *in vitro* mirrored that during embryonic development. Moreover, gene expression for the most part qualitatively paralleled that seen during *bona fide* cardiac development. When maintained under typical culture conditions, ES-derived cardiomyocytes did not acquire the gross morphologic attributes, or the quantitative levels of gene expression, characteristic of neonatal

or adult cardiomyocytes. However, since the introduction of ES cells into tetraploid blastocysts resulted in the generation of entirely stem cell-derived mice with normal hearts, murine ES cells clearly undergo *bona fide* cardiomyogenic differentiation when placed in the appropriate developmental environment. Thus, the differentiated status of ES-derived cardiomyocytes observed *in vitro* likely reflected limitations in the cell culture conditions. It remains to be seen if utilization of tissue bioengineering approaches that enhance fetal cardiomyocyte differentiation *in vitro* will have a similar effect on ES-derived cardiomyocytes.

The ability to form multiple tissue lineages following transplantation is a hallmark of pluripotent ES cells. Indeed, transplantation of undifferentiated ES cells into healthy or damaged hearts resulted in teratoma formation. Accordingly, the use of ES cells to treat heart disease would require *in vitro* differentiation and purification of donor cardiomyocytes (or alternatively, committed cardiomyogenic precursors) prior to transplantation. Unfortunately, cardiomyocytes typically comprise only 0.5% to circa 5% of the cells present in randomly differentiating EBs. Considerable effort has thus been invested to identify methodologies to enhance cardiomyogenic yield during *in vitro* differentiation.

Early efforts relied on genetic modification of the progenitor cells, such that the resulting cardiomyocytes could be separated from non-myocytes (Figure 56-2). For example, undifferentiated ES cells carrying a transgene comprising the cardiac-restricted alpha-myosin heavy chain promoter driving expression of a cDNA encoding aminoglycoside phosphotransferase were allowed to differentiate *in vitro*. After cardiomyogenic differentiation was apparent (as evidenced by the appearance of spontaneous contractile activity), the cultures were treated with G418. Since the myosin heavy chain promoter was only active in cardiomyocytes, only those cells expressed aminoglycoside phosphotransferase and survived G418 treatment. Consequently, virtually all of the non-myocytes were eliminated from the culture. A similar approach was used to isolate cardiomyocytes by virtue of targeted expression of EGFP, followed by fluorescence activated cell sorting (FACS). Both approaches have been used to isolate human ES-derived cardiomyocytes.

Additional strategies to enhance cardiomyogenic yield have been developed. Of note, Murry and colleagues demonstrated that monolayer cultures of human ES undergo high levels of cardiomyogenic induction when treated with activin A and bone morphogenetic protein-4. Following density centrifugation, an average cardiomyocyte content of 82% was obtained. Using a different approach, Keller and colleagues noted that cardiomyogenic progenitors transiently expressed vascular endothelial growth factor receptor 2. These progenitors could be isolated by sequential rounds of FACS. Remarkably, cardiomyocytes, vascular smooth muscle cells, and endothelial cells were obtained following *in vitro* or *in vivo* differentiation, suggesting the presence of a common progenitor cell for these three cardiovascular cell lineages. Similar progenitors have been obtained based on other markers, and also from human ES cells.

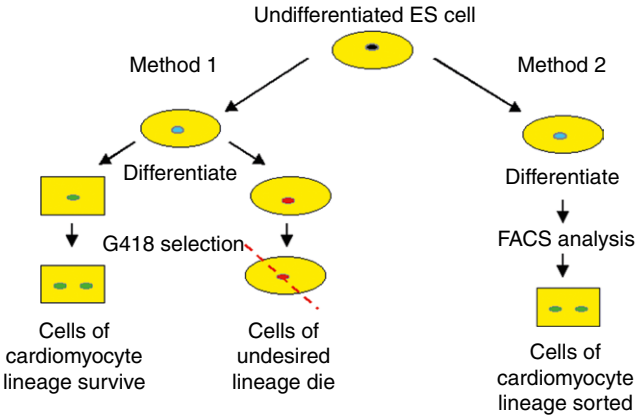


FIGURE 56-2 Selection scheme to produce pure ES-derived cardiomyocyte cultures. The cartoon illustrates two methods to separate ES-derived cardiomyocytes from non-myocytes, by virtue of cardiac-specific expression of a transgene. In Method 1, undifferentiated ES cells carrying a transgene comprising the cardiac-restricted alpha-myosin heavy chain promoter driving expression of aminoglycoside phosphotransferase were allowed to differentiate *in vitro*. Cultures were treated with G418 following cardiomyogenic differentiation to select for cardiomyocytes, since the transgene promoter was only active in cardiomyocytes. In Method 2, undifferentiated ES cells were transfected with a transgene comprising the ventricular-specific myosin light chain-2v (MLC-2v) promoter driving expression of enhanced green fluorescent protein (EGFP), and allowed to differentiate *in vitro*. Following cardiomyogenic differentiation, FACS was used to isolate a cardiomyocyte pure population.

TRANSPLANTATION OF EMBRYONIC STEM CELL-DERIVED CARDIOMYOCYTES IN PRECLINICAL STUDIES, AND CHALLENGES FOR THEIR CLINICAL IMPLEMENTATION

To date, numerous studies have been reported wherein ES cells or ES-derived cardiomyocytes were transplanted into the heart of experimental animals, on some occasions with contradictory results. The initial study transplanted mouse ES-derived cardiomyocytes (generated using the G418 selection scheme described above) into the hearts of adult mice. The donor cardiomyocytes formed stable grafts in the recipient heart, and were well aligned with host cardiomyocytes. Importantly, teratomas were not observed in this study, indicating that the purification scheme efficiently removed any residual multipotent cells from the differentiating ES cultures. Numerous studies have also transplanted human ES-derived cardiomyocytes into recipient hearts. Figure 56-3 shows the results from a study in which human ES-derived cardiomyocytes expressing an EGFP reporter transgene were transplanted into rat hearts following ischemia/reperfusion injury with minimal infarction. The hearts were analyzed four weeks post-transplantation. The

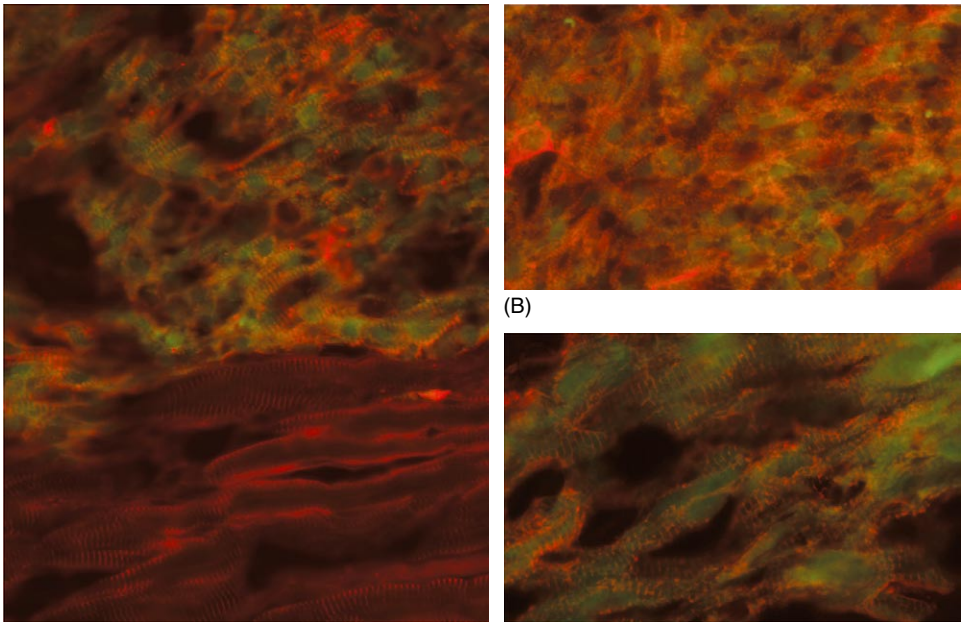


FIGURE 56-3 Stable engraftment of EGFP-expressing human ES-derived cardiomyocytes in rat hearts. Merged images of EGFP (green; epi-fluorescent signal) and anti-cardiac alpha-actinin immune reactivity (red; rhodamine-conjugated secondary antibody) are shown. Note the presence of organized myofibers within the human donor cells.

sections were stained with an anti-cardiac alpha-actinin antibody (rhodamine-conjugated secondary antibody, red signal). Panel A shows the border between the donor human ES-derived myocardium (upper region, donor cells exhibit green epi-fluorescence), and the host rat myocardium (lower region, no green signal). Panels B and C show regions within the donor cell graft. Myofibers in the human ES-derived cardiomyocytes were readily detected (in some cases, the myofibers were highly organized, as in Panel C).

Given their cardiomyogenic potential, it is not surprising that a large number of studies have tested undifferentiated ES cells, ES-derived cardiomyogenic progenitors, or ES-derived cardiomyocytes for their ability to improve cardiac function following transplantation into injured hearts. Typically, animals were subjected to some form of experimental cardiac injury. Control groups were injected with the appropriate vehicle, or alternatively non-cardiomyogenic cell types, while experimental groups were injected with the cell population being tested. Cardiac function and structure were then monitored at subsequent time points. The control groups exhibited a progressive deterioration in cardiac function and structure, commensurate with the degree of myocardial injury. This progressive deterioration was often lessened in the experimental groups receiving cardiomyogenic donor cells.

While quite promising, these studies also highlight the current limitations encountered with cell transplantation-based interventions. Most studies failed to ascertain if the donor cells were functionally coupled to the host myocardium following transplantation. This is an important caveat, particularly when transplanting into injured hearts, as the donor cardiomyocytes were often surrounded by fibrous tissue (and thus were less likely to be electrically coupled to the remote myocardium). Moreover, the impact on cardiac function was often disproportionate to the size of donor cell grafts (which were typically quite small). Given this, indirect mechanisms were likely to have contributed to the improved cardiac function in these studies. For example, cell transplantation could have rescued at-risk myocardium, enhanced healing of the infarct, and/or altered adverse post-injury remodeling, via paracrine-based mechanisms similar to those proposed for hearts transplanted with non-cardiomyogenic stem cells. Presently it is not clear if donor cardiomyocytes can repopulate the heart at sufficient density to directly impact cardiac function. This limitation, as well as a number of additional obstacles, must be overcome for clinical implementation of ES-derived cardiomyocyte transplantation. These are considered below.

Overcoming Limitations in Graft Size

The relatively low levels of donor cell seeding in the host myocardium, particularly following engraftment into injured hearts, is a major limitation of current cardiomyocyte transplantation protocols. Using fetal cardiomyocytes as donor cells, several groups demonstrated that the preponderance of donor cardiomyocytes die following transplantation into the heart, with typically less than 5% of the cells successfully seeding the myocardium. Interventions aimed at enhancing donor cell survival, seeding, and/or post-transplantation proliferation are required to achieve transmural myocardial replacement in injured hearts. Towards that end, Murry and colleagues recently developed a combinatorial approach, wherein donor cells were pre-treated with reagents aimed at blocking ischemia-, anoikis-, apoptosis-, and

inflammation-mediated cell death. Pre-treatment of human ES-derived cardiomyocytes with these reagents markedly enhanced donor cell survival following transplantation into rat hearts with ischemia/reperfusion injury.

Even with this pro-survival pre-treatment regimen, cell transplantation only resulted in remuscularization of, on average, 4% of the infarct (corresponding to only 0.4% of the left ventricle mass). Additional interventions will thus be required to achieve a more extensive (i.e., transmural) remuscularization. Towards that end, numerous cardiomyocyte pro-survival pathways have been identified. Moreover, pathways that impact on cardiomyocyte proliferation during development, and in some cases result in cell cycle re-entry in adult cardiomyocytes, have been identified. Genetic modification of the ES cells, or alternatively development of small molecule modulators of the genetic pathways in question, could be exploited to enhance seeding efficiencies and/or graft size. Use of committed progenitors might also enhance graft size, provided that they exhibit greater post-transplantation proliferation compared with more mature donor cells. Delivery of donor cells in the context of *in vitro* engineered tissue constructs constitutes another viable alternative to enhance graft size. Relatively large volumes of engraftment in infarcted hearts were obtained by surgical attachment of experimental heart tissue which was generated *in vitro* by seeding collagen-based casts with neonatal rat cardiomyocytes. Interestingly, collagen-based casts with complex replacement structures were easily-generated, and appear to undergo rapid vascularization following surgical attachment. These approaches, alone or in combination, may help achieve transmural remuscularization of infarcted hearts with ES-derived cardiomyocytes.

Overcoming Limitations in Donor Cell Production

The ability to generate clinically relevant cell numbers constitutes another significant hurdle for implementation of clinical applications with ES-derived cardiomyocytes. In this context it is interesting to make some projections of cell number requirements. It has been estimated that the left ventricle of the human heart contains about 6×10^9 cardiomyocytes. Consequently, cell transplantation therapies aimed at replacing tissues lost to large myocardial infarction could require the successful incorporation of as many as 3×10^9 donor cardiomyocytes. Generation of this number of donor cells, with concomitant elimination of contaminating cells with teratoma potential, is a formidable task. While the identification of cardiomyogenic growth factors described above will greatly facilitate this process, large-scale production of donor cells will almost certainly require transition from two-dimensional tissue culture-based approaches, to three-dimensional bioreactor-based approaches.

The main challenge to achieve large-scale generation of ES-derived cells is to establish conditions that would initially support the amplification and initial differentiation of ES cells (as for example via EBs formation), and which on subsequent culture would support the further growth and enrichment of the desired cell type. This is particularly challenging, due to the fact that characteristics of the ES cells (and their derivatives) change continuously during the differentiation process. A single-step culture protocol to generate ES-derived cardiomyocytes in an actively-aerated, fully-controlled tank reactor can accomplish these tasks. It utilized a common two-liter bioreactor tank equipped with a blade impeller creating axial flow, coupled with the G418 genetic selection scheme described above. The bioreactor was initially seeded with undifferentiated ES cells. After nine days of culture under conditions suitable for cell expansion, EB formation and differentiation, G418 was added to eliminate the non-cardiomyocytes. After nine days of culture in the presence of G418, more than 109 cardiomyocytes (>99% pure) were obtained from a two-liter reaction vessel. Although quite preliminary, this type of approach provided cell numbers nearing those which are likely to be required for clinical applications.

Overcoming Donor Cell Immune Rejection

Issues associated with the use of allogenic donor cells constitute an additional hurdle for the widespread application of ES-derived cardiomyocyte transplantation. Towards this end, the development of comparably mild immune suppression protocols, used for type-1 diabetic patients following cadaveric beta-cell transplantation, are quite encouraging. Hope, in this regard, is bolstered by the observation that ES-derived cardiomyocytes did not appear to undergo acute rejection when transplanted into rat hearts with experimental infarctions. Moreover, a novel system based on ablation of IL-15 responsive T-cells might ultimately be useful to induce tolerance to allogenic ES-derived donor cells.

The generation of autologous donor cells would bypass immune suppression/rejection concerns. Although cumbersome, nuclear transfer has been successfully used to generate autologous ES-like cells, which could be used to generate ES-derived cardiomyocytes that would escape immune rejection. More recently, Yamanaka and colleagues demonstrated that transient expression of four gene products was sufficient to reprogram transduced fibroblasts into induced pluripotent stem (iPS) cells. Differentiation of iPS cells *in vitro* resulted in the formation of *bona fide* cardiomyocytes. Further refinement of iPS technology (in particular, the ability to generate iPS cells via cytokine treatment, rather than via transgenesis) would constitute an ideal source of donor cells. Indeed, some cells are able to undergo reprogramming into ES-like cells. For example,

Hasenfuss and colleagues demonstrated that spermatogonial stem cells spontaneously reprogrammed into ES-like cells during *in vitro* culture. When induced to differentiate, these ES-like cells gave rise to *bona fide* cardiomyocytes. Thus, there appear to be multiple approaches with which to generate autologous ES-like cells suitable for generation of donor cardiomyocytes. The additional time requirements for the reprogramming event and validation of ES-like characteristics constitute an additional challenge for the clinical implementation of such tailor-made autologous donor cells.

SUMMARY

The potential use of cellular transplantation for the treatment of heart disease is gaining greater acceptance. The notion of using stem cell-derived, and in particular ES-derived, donor cardiomyocytes for partial restoration of lost cardiac mass in diseased hearts has been validated in experimental animals. Additional research is required to determine if it is possible to seed sufficient numbers of donor cells to directly impact on cardiac function, and also to develop viable strategies to prevent donor cell rejection. If these latter hurdles are overcome, cellular transplantation with ES-derived cardiomyocytes will emerge as a promising intervention for the treatment of heart disease.

ACKNOWLEDGMENTS

We thank the NHLBI for support (MR & LJF).

KEY WORDS

Cardiomyocytes Muscle cells in the heart which are interconnected via gap junctions and which participate in a functional syncytium.

Embryoid bodies Three-dimensional structures obtained when ES cells are allowed to differentiate while grown in suspension culture.

Embryonic stem (ES) cells Multipotent cells derived from the inner cell mass of blastocysts and able to differentiate under appropriate conditions into cells of ecto-, endo-, and mesodermal origin.

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Chapter 57

Insulin-producing Cells Derived from Stem Cells: A Potential Treatment for Diabetes

Susan Bonner-Weir and Gordon C. Weir

THE NEED FOR INSULIN-PRODUCING CELLS

The importance of β-cell replacement therapy

Although insulin was discovered over 75 years ago, the complications of diabetes still produce devastating consequences. The link between high blood glucose levels and the complications of retinopathy, nephropathy, and neuropathy is now established beyond doubt. An obvious path to prevention of complications is some form of β-cell replacement therapy. Since 1978, over 15 000 pancreas transplants have been performed, and now over 1000 are done yearly, but these require major surgery. It would be preferable to transplant only the pancreatic islet cells, which comprise only about 1–2% of the pancreas, but islet transplantation, lagging behind whole organ transplants, only began to obtain encouraging results in the late 1980s. Generally poor outcomes were obtained throughout the 1990s, but the introduction of the Edmonton protocol in 2000 provided far better results, the improvement being due to better islet preparations, transplantation of more islets, and improved immunosuppression. Islets are introduced into the liver through the portal vein via transhepatic angiography. These important studies provide proof-of-principle for the concept of cellular transplants as a treatment for diabetes. Nonetheless, the expectations from the Edmonton results have not met expectations. While recipients are happy about being insulin independent, and greatly benefit by being free of hypoglycemia, graft function is not durable; most of those who became insulin independent are back on insulin within two years. Moreover, the side-effects of the procedures and immunosuppression bring danger and discomfort. While results are improving in the relatively small number of centers doing clinical trials, it is clear that much must be done to bring β-cell replacement to all in need.

Limited Supplies of Insulin-producing Cells

In spite of this success, the supply of insulin-producing pancreatic β-cells, which is currently only from cadaver donors, is insufficient, and limits the extension of this therapy. In the United States, it would be a major challenge to obtain 3000 usable cadaver pancreases per year. Much attention is focused on finding new sources of insulin-producing cells that can be used for transplantation, or finding that islet regeneration can be induced. The quest includes exploring the potential of embryonic and adult stem cells, transdifferentiation such as directing acinar cells or hepatocytes to make insulin, altering cells with bioengineering, developing human cell lines, and the use of β-cells from other species as xenografts.

DEFINING β-CELLS, STEM CELLS, AND PROGENITOR CELLS

For the purposes of this chapter, a β-cell is defined as a cell with the phenotype of a mature insulin-producing cell found in pancreatic islets. It is possible to have insulin-producing cells that are immature and lack the full phenotype of a true β-cell. Some of these are β-cell precursors that, at some point, can be called young β-cells. However, there are cells containing insulin that will never become β-cells, such as those identified in the thymus, brain, retina, liver, and yolk sac.

It is also necessary to distinguish between the words "stem," "progenitor," and "precursor" cells. All new insulin-producing cells originate from precursor cells, which are

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not necessarily true stem cells. Stem cells can be defined as precursor cells capable of indefinite self-renewal. An embryonic stem cell (ESC) is pluripotent, being capable to differentiate into the three embryonic germ layers, ectoderm, endoderm, and mesoderm, and then any cell type of the body. Typical adult stem cells, such as are found in bone marrow, intestine, and skin, are multipotent, being able to form a variety of cell types restricted to a defined lineage. For example, cells originating from bone marrow stem cells are of mesodermal origin, and form blood cell elements and endothelial cells. Then there are precursor cells with more restricted products, such as hepatic oval cells that can form either new hepatocytes or bile duct epithelia. Facultative progenitor cells also include pancreatic duct cells that can form pancreatic acini and islets.

THE POTENTIAL OF EMBRYONIC STEM CELLS AS A SOURCE OF INSULIN-PRODUCING CELLS

As a guide for exploiting the potential of human embryonic stem cells (hESCs) to make pancreatic β-cells, mouse ESCs have become an important model. With their potential to develop into virtually any cell type, they can be kept in an undifferentiated state being grown on feeder layers of irradiated mouse embryonic fibroblasts with leukemia inhibitory factor (LIF). To use hESCs for clinical purposes it will probably be necessary to grow them on something other than mouse cells, which might include gelatin or human fibroblasts. Once removed from feeder layers, ESCs develop embryoid bodies, expressing their pluripotency to develop the three germlines, ectoderm, endoderm, and mesoderm. Another potentially useful source of pluripotent cells is embryonic germ cells derived from primordial gonads. There is also a report of pluripotent cells being derived from adult bone marrow cells. Similar multipotency has been found in cells obtained from amniotic fluid.

Another recent major advance with potential benefit for diabetes is a method for producing pluripotent stem cells from fully-differentiated cells. Yamanaka and coworkers found that the introduction of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) into mouse fibroblasts could reprogram the cells to an undifferentiated state similar to ESCs. These cells, which are called induced pluripotent stem (iPS) cells, may be able to make pancreatic islet cells, and will not face the same political hurdles as human embryonic stem cells. Compared with somatic cell nuclear transfer technology, this may be a much more convenient way for a transplant recipient to obtain genetically-identical islet cells.

Directing Differentiation

ESCs can be directed toward different progeny by a variety of factors, including soluble growth and differentiation mediators, matrix materials, and cell–cell contacts. Using a five-stage approach, McKay and colleagues were able to direct mouse ESCs to cells with islet cell markers. Another advance came from Keller *et al.* with the demonstration that mouse ESCs can be pushed to express endoderm markers by culture in the presence of activin A, using serum-free conditions. Another approach to direct differentiation is to use markers of various stages of development, coupled with high throughput screening, to identify small molecules or other factors that will change the frequency of such markers. For example, to direct ESC towards β-cells, fluorescent markers of definitive endoderm or pancreatic precursor cells are proving to be valuable.

Key Factors Involved in Pancreatic and Islet Development

Knowledge of pancreatic and islet differentiation provides important help for work with ESCs. The finding that Pdx1 knockouts have virtually no pancreatic development identified a key early role for this transcription factor, which is different from its role at a later stage in maintaining β-cell differentiation. Another key point is that Ngn3 is expressed in precursor cells committed to differentiating into islets. Transient Ngn3 expression is thought to be induced by a combination of inhibition of notch signaling and stimulation of signaling by molecules of the TGF-β family. Pax4 appears to play a key role in post-Ngn3 β-cell differentiation. Knockout of Pax4 leads to an increased α-cell number, but no β- or delta-cells. Other transcription factors important for β-cell development are BETA2/NeuroD, Nkx2.2, Nkx6.1, and MafA, which seems to be especially important for the final stages of β cell differentiation.

Generation of Insulin-producing Cells from Mouse and Human Embryonic Stem Cells

The earliest efforts to direct ESC differentiation were mostly done with murine ESCs. Different groups used elaborate multi-stage protocols, which resulted in the production of cells that had some β-cell characteristics, but fell short of being true fully-developed cells. The first work with hESCs provided evidence for some insulin production, but there was little characterization of the cells.

Employing knowledge of pancreatic development and lessons from work on mouse ESC, Novocell Inc. applied a systematic approach to human ESC, and has had striking success. Each of five stages employed particular culture conditions and added differentiation factors. These stages and their differentiation factors included the following:

Stage 1 Definitive Endoderm (activin and Wnt signaling);
Stage 2 Primitive Foregut (epidermal growth factor and fibroblast growth factor 10 (FGF 10);

Stage 3 Posterior Foregut (retinoic acid, cyclopamine);
Stage 4 Pancreatic Endoderm and Endocrine Precursors (γ-secretase inhibitor, exendin 4); and
Stage 5 Hormone Expressing Endocrine Cells (exendin-4, IGF-1, hepatocyte growth factor).

With this protocol, cells containing insulin, glucagon, somatostatin, and pancreatic polypeptide were produced. Importantly, the insulin content of these cells was far higher than had been achieved before. However, glucose-stimulated insulin secretion (GSIS) could not be demonstrated, indicating that the insulin-containing cells were immature. Other groups have also had success in directing hESCs towards β-cells.

The next step for Novocell was to make a few changes in the differentiation protocol and transplant the pancreatic precursor cells (Stage 4) into immunocompromised mice. After these cells are placed into an *in vivo* transplant site, some develop into fully mature functional β-cells. They contained all of the β-cell markers that were examined including MafA by electron microscopy; they contained characteristic β-cell granules with dense cores and halos; they efficiently converted proinsulin to insulin; and they had GSIS. In addition, they could normalize the glucose levels of streptozocin (STZ) diabetes. This experiment was made possible because STZ selectively kills mouse β-cells, but not the human β-cells in a graft site. The site of the grafts did not make much difference; subcutaneous, renal subcapsule, and epididymal fat pad sites were all successful. All of the other pancreatic endocrine cell types were found, but the grafts also contained many presumably immature cells. Importantly, of the 46 grafts examined by pathologists, one contained a teratoma, and six others had teratomatous elements. This raises important concerns about the safety of transplanting precursor cells into humans with diabetes. However, at this stage of the work, no effort was made to eliminate immature cells with teratoma formation potential. Certainly it will be a high priority to find ways to obtain a population of fully-mature cells *in vitro*, or to obtain a population of transplantable precursor cells that are far enough developed to not form teratomas.

Need for Rigorous Assessment of Insulin-producing Cells Derived from Embryonic Stem Cells or induced Pluripotent Stem Cells

It is essential that future studies rigorously compare ESC or iPS cell-derived cells with normal β-cells, both in culture and in a transplant graft. The cells should be assessed for insulin content; insulin secretory response to see if a normal glucose dose response curve is obtained; EM characterization of granules to see if dense core granules are present; characteristic staining for IAPP; proinsulin conversion to insulin and C-peptide; and, finally, gene expression profiles with a variety of techniques including gene arrays. There are many examples of cells being generated that have β-cell markers and some insulin, but there is increasing reason to think that they must be very similar to true mature β-cells to function adequately in a transplant situation.

Relationships between Islet Cells in Transplant Grafts, and Importance for Function

When developing strategies to transplant islet cells derived from ESCs, iPS cells, or other sources, it is important to consider the complexities of transplanting even normal islets, and the lessons learned. The pancreatic islet is a highly-organized micro-organ, in which β-cells are typically found in the core, with non-β-cells forming a surrounding mantle. The blood supply comes from arterioles that penetrate gaps in the islet mantle and then form a glomerular-like network of capillaries in the islet core. The capillaries leave an islet through the mantle to enter the acinar tissue to form the islet-acinar portal system. The peptides produced by the different islet cells have the capacity to influence other islet cells: for example, glucagon can stimulate the secretion of insulin and somatostatin; somatostatin can inhibit glucagon and insulin secretion; and insulin can inhibit glucagon secretion. In spite of this potential, some of these interactions probably never take place *in vivo*. As fits with the known vascular pattern of islets, the upstream β-cells of the central core are probably protected from the potentially powerful effects of glucagon and somatostatin, because blood and interstitial fluid flow from core to mantle. In contrast, the mantle cells, being downstream, are bathed in high concentrations of insulin, such that glucagon suppression by glucose appears to some extent indirectly mediated by local insulin secretion. Ingenious physiological experiments by Samols and Stagner indicate that these intraislet interactions are unidirectional, B to A to D (B–A–D), these referring to β, α, and δ cells, respectively. Additional data supporting this concept have been generated by the group of Schuit *et al.* using a glucagon antagonist.

It has been suggested that non-β-cells are randomly distributed throughout the core of human islets, in contrast to the β-cell core and the non-β-cell mantle pattern uniformly seen in rodent islets. However, while it is true that non-β-cells are found in the core, the distribution is not random. Human islets are typically made up of subunits, in which β-cells form a core with surrounding non-β-cells. While the vascular patterns of human islets have not been elucidated, the physiological relationship between β-cells and non-β-cells in human islets is essentially the same as that seen in rodents.

Within islet grafts, the relationships between the different islet cell types appear to be disrupted, but they are not well-understood. It is clear that substantial remodeling of transplanted islets occurs during the early days to weeks of the transplant. The vascular pattern of the new microvasculature does not faithfully recapitulate the pre-isolation pattern. Islets in the pancreas are richly vascularized by capillaries with fenestrated endothelium, but islet grafts have a much less dense vasculature, which is no doubt a factor in grafts having an oxygen tension of about 5 mm Hg, compared with 40 mg Hg in endogenous islets, as shown by Carlsson *et al.* A key point is that the *in vivo* B–A–D relationship is completely disrupted when islets are isolated, and no doubt is not fully re-established after transplantation, which could allow β-cells to be locally influenced by α-cells.

If α-cells were upstream in a graft, the local glucagon secretion could potentate insulin secretion with a beneficial effect. On the other hand, it could lower the set point for GSIS by potentiating the stimulatory effect of normal concentrations of glucose, and thus possibly cause hypoglycemia. We have found that rats transplanted with islets in the liver become hypoglycemic with exercise. Hypoglycemia has not been described with human transplants, but may become an issue when it is possible to transplant a larger islet mass. There are, however, potential benefits from antiapoptotic effects of local glucagon on β-cells. GLP-1 signaling, which is similar to that of glucagon, has an antiapoptotic influence on β-cells, and this effect seems likely to be exerted through cyclic-AMP, IRS-2, and Akt.

It is important to know if pure insulin-producing cells derived from ESC will function appropriately when transplanted without non-β-cells. Based upon the B–A–D concept, our bias is that β-cells of the pancreas normally function well without ever seeing the secretory products from the mantle. Valuable studies have been performed using flow cytometry to sort highly enriched populations of β-cells on the basis of endogenous fluorescence. One group found that transplantation into rat livers of aggregated islet cells with fewer non-β-cells were a little less durable than intact islets. Our own studies concluded that rat islet cell aggregates depleted of non-β-cells transplanted under the kidney capsule of diabetic immunocompromised mice had excellent long-term function. These studies provide some assurance that ESC- or iPS cell-derived β-cells will function well when transplanted without islet non-β-cells, but there are still many questions that remain. What will be the ideal site for the transplantation of such cells? An obvious approach would be for the cells to be aggregated *in vitro*, and then placed into the liver via the portal vein. Other candidate sites might include the peritoneal cavity, a renal subcapsular site, a subcutaneous pocket, or even the pancreas.

What Immunological Barriers Will be Faced by Transplanted Insulin-producing Cells Derived from Embryonic Stem Cells or Induced Pluripotent Stem Cells?

The prospect of being able to use iPS technology to derive islet cells from diabetics in need of β-cell replacement is very exciting. For subjects with type 1 diabetes, allograft rejection would no longer be an issue, but autoimmunity would still need to be controlled. Although not widely-appreciated, subjects with type 2 diabetes have reduction of β-cell mass to 30–60% of normal; they too will become normoglycemic with β-cell replacement. If islet cells are derived from their own iPS cells, there should be no problems with immune destruction. If cells are derived from ESCs, there should be opportunities for tissue matching, which will be helpful, but it is expected that immunosuppression, with its daunting toxicity, will still be required. Improvements in the tolerability of these drugs should occur with time, and eventually the goals of transplantation tolerance and safe control of autoimmunity should be realized. However, because control of autoimmunity may not be achieved soon, it may be necessary to use immunobarrier technology to protect aggregates of insulin-producing cells. One approach has been to encapsulate islets in small gel beads using alginate. The concept is over 30 years' old, and even though there has been impressive success in rodents, bringing this approach to large animals or humans has proved very difficult. Nonetheless, work continues, and prospects for the development of more effective biomaterials and strategies are encouraging.

Conclusions about Embryonic Stem Cells and Induced Pluripotent Stem Cells

For the purposes of making insulin-producing cells that are true β-cells, the potential of ESCs, and probably iPS cells, is tantalizing, especially considering the clinical need for cells that could solve the problem of diabetes. The impressive progress within the past five years can be expected to intensify efforts to find ways to bring derivatives of these pluripotent cells to the clinic.

THE POTENTIAL OF ADULT STEM/PROGENITOR CELLS AS A SOURCE OF INSULIN-PRODUCING CELLS

New β-cells are Formed Throughout Adult Life

Maintenance of β-cell mass is a dynamic process of continuing apoptosis that is balanced by the formation of new β-cells by replication of existing β-cells and neogenesis,

which is the formation of new islets from precursor cells. The neonatal period in rodents is characterized by very active β-cell replication and neogenesis, and near the time of weaning there is an increase in β-cell apoptosis that leads to a remodeling of the endocrine pancreas. Through measurement of rates of β-cell replication, cell size and β-cell mass, and making assumptions about neogenesis and apoptosis, we have estimated the lifetime of a rat β-cell as approximately 58 days. It is more difficult to make estimates of the life span of human β-cells, but based on low replication rates, as determined by Ki67, and the low incidence of apoptosis, as judged by TUNEL staining, it seems likely that they can live for many years. A major variable in question is neogenesis, which could be substantial in humans.

The endocrine pancreas of rats has considerable capacity for regeneration, as shown by our studies with 90% partial pancreatectomy. Even by four weeks following this surgical reduction in β-cell mass, the β-cell mass has increased from 10% to 42% of controls through the combined contributions of β-cell replication and neogenesis. There seem to be important species differences in how β-cell mass expands. Mice with genetically induced insulin resistance develop gigantic islets, and a marked increase in β-cell mass that can reach 20% of pancreatic volume, mainly through β-cell replication. Humans with the insulin resistance of obesity also have increased β-cell mass, but this increase is only about 30–50% compared with lean subjects, moreover, the islets are not particularly enlarged (SBW, unpublished). The frequent finding of insulin-positive clusters of cells appearing to emerge from pancreatic ducts in adult pancreases, and the very low rate of β-cell replication as judged by staining with Ki67, suggest that neogenesis is an important contributor to the increase in mass in adult humans. Another mechanism for increasing β-cell mass, β-cell hypertrophy (increased individual cell volume), has been found in rats in a variety of situations, including glucose infusions, pregnancy, and partial pancreatectomy.

Debate about β-cell Replication Versus Neogenesis

Although it is clear that β-cells are formed in adult pancreas, the source of these new cells has been vigorously debated. A 2004 study by Dor *et al.* used lineage-tracing with the insulin promoter in mice to conclude that no new islets were formed after birth or after 70% pancreatectomy, but that new β-cells could be formed by replication of pre-existing β-cells. Other studies using different tools have supported this view. These studies are likely correct in the observation that β-cell replication is the dominant mechanism for β-cell expansion in adult mice, but they do not convincingly show that new islets are not formed during the neonatal life or after partial pancreatectomy. These striking claims fly in the face of decades of observation and circumstantial evidence that new islets bud from ducts during adult life.

Arguments Favoring the Ductal Origin of New Islets

Morphologically, new islets appear to bud from the ducts, breaking through the epithelial basement lamina and migrating away from the duct. The process in the adult pancreas closely resembles new islet formation in the embryonic pancreas, which is also seen as budding off from duct-like structures. Similar differentiation of islet cells from adult duct tissue was shown experimentally by Dudek *et al.*, with the finding of cells containing islet hormones after transplantation of adult pancreatic ducts and fetal mesenchyme. In rat insulin promoter (RIP) interferon-γ transgenic mice, newly-formed islets were often seen within the lumen of the ducts, again suggesting their origin from ducts.

Other evidence supporting the concept is that after partial pancreatectomy in rats, we have found that ductal cells rapidly replicate and dedifferentiate, with a marked increase in PDX-1 protein, a factor known to be important for pancreas development and β-cell function. This change to a less-differentiated phenotype precedes the differentiation of whole new lobes of pancreas. Yet another finding strengthening this hypothesis is the marked change in composition of porcine neonatal pancreatic cell clusters transplanted under the kidney capsule of immunodeficient mice. When transplanted, the majority of cells can be stained with the duct marker cytokeratin 7, but after several months 94% of the cells were β-cells. During this engraftment, as well as in neonatal pancreas, cells co-stained for both insulin and cytokeratin 7 could be found, suggesting the presence of residual duct markers as duct cells become β-cells. This finding is extended by gene profiling with microarrays of new versus mature islets seven days after partial pancreatectomy in rats. These new islets, which can be identified by markers of new lobe formation, are about three days of age. Their β-cells contain a variety of duct markers, supporting the duct precursor hypothesis.

These findings evolved into the hypothesis that islets are derived from differentiated duct cells in the pancreas, which can serve as facultative multipotent progenitor cells. Particularly suggestive has been our finding that human duct cells in tissue culture can form cysts from which budding islet cells emerge; these have been called cultivated human islet buds (CHIBs). These findings have been confirmed by others. Further evidence of the duct cells being precursors is supported by the finding that duct cells purified with antibodies recognizing the duct cell surface antigen CA 19.9, which are transplanted under the kidney capsule of

immunocompromised mice, can generate insulin-containing cells. These results are complemented by other work showing that human non-endocrine epithelial cells undergo similar differentiation in a transplant situation.

In spite of such strong circumstantial evidence, some will not accept the duct cell origin hypothesis without rigorous lineage-tracing experiments. This has now been accomplished by our studies marking duct cell progeny with conditionally-expressed carbonic anhydrase II (CA II) Cre transgenic mice, in which cells are marked with β-galactosidase. By following the appearance of marked cells, it seems virtually certain that duct cells expressing CA II are the source of quantitatively important new endocrine and exocrine pancreatic elements during the neonatal period and after pancreatic duct ligation. This work is complemented by the finding by the Heimberg group, that cells marked during adult life by the expression of Ngn3 can form all islet cell types after duct ligation. All newly-formed pancreatic islet cells are known to originate from precursor cells that express Ngn3. While the Heimberg experiments do not prove that the duct cell origin hypothesis, the precursor cells are found in ducts. Work from these two groups has now convincingly shown with lineage-tracing that new β-cells are formed in the adult mouse pancreas.

A hypothetical argument supporting the concept of duct cells serving as facultative precursor cells comes from estimating how many precursor cells must be needed to form a new islet. After partial pancreatectomy in rats, fully-formed new islets can be seen by 72 hours after surgery, with these new islets consisting of 1000–1500 cells. No increased replication in the pancreas is seen until 24 hours after surgery, so they must form within 48 hours. With cell cycle times in rodents being 10–20 hours, 16 starting cells with a doubling time of 10 hours (5 doublings over 48 hours), would be required to make 1000 cells. If the doubling time were 12 hours, then 64 cells would be required. Because there are so few candidate non-duct precursor cells that can be found morphologically, these calculations fit with the local abundance of duct precursor cells, and the presence of hormone-positive cells within the basement membrane of ducts. Moreover, this concept fits with the demonstration that β-cells in islets are of polyclonal origin.

The possibility that pancreatic duct cells can be exploited to generate more β-cells continues to be of great interest. One possibility is that duct cells might be expanded *in vitro*, and then stimulated to form β-cells that could be used for transplantation. Another possibility is that duct cells might be stimulated to form islets *in vivo*, such that new β-cells might be produced, either in the native pancreas, or in a graft site from duct cells contained within transplanted impure human islet preparations. There is much work now on drugs that might singly or in combination stimulate neogenesis, including glucagon-like peptide-1 (GLP-1) receptor agonists, gastrin, and epidermal growth factor. Evidence compatible with endogenous regeneration comes from some subjects undergoing gastric bypass surgery who develop such severe hypoglycemia that partial pancreatectomies must be performed. Marked increases in β-cell mass and the appearance of neogenesis have been found in these pancreases. GLP-1 has been implicated, because of the very high circulating levels found in these individuals.

Pancreatic Non-duct Cell Candidate Precursor Cells

Other candidate precursor cells have been found in the pancreas. Two groups have found single cells from mouse pancreas that can form from clonal aggregates in culture, which can be differentiated to express β-cell markers, and even some insulin. These cell aggregates also express some stem cell markers. In addition, very small embryonic-like (VSEL) stem cells have been found in pancreas, which also express islet markers. Thus, these pancreatic non-duct cells have developmental similarity to islets, however, it remains to be seen if they can become fully-differentiated to islet cells, or are quantitatively sufficient. Perhaps they sustain a pool of ductal progenitors. The duct cells, which have a fully-differentiated phenotype, appear to be a major source of neogenesis, are not true stem cells in having the capacity for self-renewal or expressing the usual markers. The search for a true pancreatic stem cell will continue, but the prospects for finding a source that will be clinically useful do not seem high.

The Postulate that β-cells can Become Islet Precursor Cells

Because of the intense interest in identifying islet precursor cells, a variety of studies have made a case for the presence of precursor cells that originate from islets. One postulate for a β-cell precursor in islets comes from streptozocin-treated rats, in which cells stained for both insulin and somatostatin are thought to differentiate into β-cells. Although these studies are all provocative and potentially important, more work is required to understand whether these are true pathways for β-cell regeneration.

A more provocative hypothesis is that β-cells have the capacity to dedifferentiate to cells that can be markedly expanded through replication, and can then differentiate back to a β-cell phenotype. There was considerable interest in the possibility that nestin, which is known to be present in neural stem cells, is involved in such a process. Work with mouse embryonic stem cells indicated that insulin-producing cells could be generated through nestin-containing precursors. It was then suggested that nestin stained cells could be derived from human β-cells; these cells had capacity for expansion and then some redifferentiation with expression of β-cell markers, but they

contained very little insulin. A great deal of debate erupted about whether nestin was involved in any way for β-cell differentiation, but the hypothesis has not yet been disproved. A related concept is that adult β-cells can serve as precursor cells by undergoing altered differentiation through an epithelial-mesenchymal transition (EMT), whereby they can be greatly expanded and then redifferentiate to express β-cell markers. However, these experiments also failed to produce significant amounts of insulin. Efrat and co-workers have provided the most convincing data that there may be a pathway to marked dedifferentiation and expansion of β-cells, and subsequent redifferentiation to fully mature β-cells, but even their success has been sporadic. There has been great interest in using lineage-tracing to show that β-cells are the true source of these expanded cells. Studies with mouse β-cells cast doubt on whether a meaningful EMT process can occur, but studies with human β-cells provide more support for such a possibility.

TRANSDIFFERENTIATION OF NON-ISLET CELLS TO ISLET CELLS

There are now a variety of studies supporting the concept that a variety of other cell types can be directed to become insulin-producing cells. Strictly speaking, the concept that differentiated duct cells can form new islets, as was discussed above, could be considered a form of transdifferentiation. Current claims about transdifferentiation now include pancreatic acinar cells, hepatocytes, and bone marrow. There were claims that splenocytes could be transdifferentiated to become β-cells, but subsequent work has not been able to confirm this supposition. The possibility that transdifferentiation within pancreatic tissues occurs is not surprising, in view of the ease with which the phenotype of islet and pancreatic cell lines can shift from one predominant gene product to another. Examples include: clonal RIN cells varying their expression of insulin, glucagon and somatostatin over time in culture; a pancreatic adenocarcinoma cell line (AR42J) that can be pushed to produce insulin by either hepatocyte growth factor or a combination of betacellulin and activin A; growing evidence that progenitor cells in adult pancreas can form hepatocytes; and that liver cells can form pancreatic cells.

Pancreatic Acinar Cell Transdifferentiation

It has been difficult to prove that differentiated adult pancreatic cells are converted to insulin-producing cells. Suggestive evidence was provided by studies in a rat duct ligation model of regeneration, in which gastrin infusions produce changes consistent with transdifferentiation of acinar cells to duct cells that can then serve as precursors for β-cell neogenesis. The same pathway for neogenesis was postulated to occur in rats receiving glucose infusions. More striking evidence for acinar transdifferentiation to islet cells has been provided by work by Melton *et al.*, in which adenoviruses expressing three transcription factors, Ngn3, Pdx-1, and MafA, injected into pancreatic parenchyma can produce new islet cells including β-cells, which partially reverse diabetes. While acinar cells can be induced to transdifferentiate to islet cells, lineage-tracing experiments indicate that such a process does not occur naturally, even in response to injury.

Bone Marrow Cells as a Source of Insulin-producing Cells

There has been much interest in the possibility that circulating bone marrow stem cells could serve as precursors for a wide variety of cells scattered throughout the body. This is complicated by the fact that bone marrow contains not only hematopoietic stem cells (HSC), but also mesenchymal stem cells. The possibility that hematopoietic stem cells (HSC) can transdifferentiate into non-hematopoietic cells has been suggested by various studies. However, this appears not to be the case, as shown in rigorous studies by Wagers *et al.* There remains the possibility that cells other than true HSC derived from bone marrow can serve as circulating stem/precursor cells, but it is unclear whether there is true transdifferentiation or cell fusion. It has recently been shown that impressive repopulation of destroyed liver tissue can be generated from bone marrow cells, but this process was clearly shown to be due to cell fusion. Nonetheless, a provocative report appeared suggesting cells from bone marrow can become glucose-responsive insulin-secreting cells in islets with this not being due to fusion, but now there are numerous careful studies indicating that such a phenomenon does not occur. Nonetheless, the question of the potential of bone marrow cells remains open, especially considering the finding by Verfaillie and coworkers that adult bone marrow cells can be pluripotent, capable of progressing to ectoderm, mesoderm, and endoderm fates. Another way in which bone marrow cells could contribute to new β-cell formation is as a facilitator rather than a precursor. In a study by Hess *et al.*, when c-kit positive adult bone marrow cells were given to mice made diabetic with streptozocin, there was evidence of regeneration of the endocrine pancreas and return to normoglycemia. Other work supports the concept that this beneficial effect is exerted by bone marrow-derived mesenchymal stem cells.

The Liver as a Source of Insulin-producing Cells

As suggested above, liver, being of endodermal origin, might be expected to be a prime candidate for transdifferentiation

to islet cells. One of the first suggestions of this premise was the finding of Ferber *et al*. that *in vivo* transduction of hepatocytes in mice with an adenovirus containing pdx1 could induce endogenous Pdx1, associated expression of other β-cell markers, and substantial amounts of insulin. A similar approach in mice employing helper-dependent adenoviruses expressing betacellulin and NeuroD appeared to result in the production of enough insulin in the liver to reverse streptozocin diabetes. Other studies provide evidence that hepatocytes can be induced to express β-cell markers, but differentiation to a true mature β-cell has yet to be demonstrated. There has also been work suggesting that hepatic oval cells can differentiate into insulin-producing cells. Another provocative finding was that human fetal liver cells transduced with human telomerase and then pdx1 produced a remarkable amount of stored and secreted insulin. These cells not only secreted insulin in a regulated manner, but also reversed diabetes when transplanted into immunodeficient diabetic mice.

Engineering Other Non-β-cells to Produce Insulin

The possibility that non-β-cells might be engineered to make insulin, and even secrete insulin in response to glucose, was pioneered by Newgard *et al.* using cultured cells. This is very challenging, because the genetic phenotype of β-cells is so complex. Now that more has been learned about the master switches that control the differentiation of cells, more promising results are beginning to emerge. Nonetheless, transplanted β-cells will almost certainly need to have a near fully-intact phenotype to provide the exquisite insulin secretory capabilities of normal β-cells required to cope with meals, fasting, and exercise. A more attractive approach may be to transform β-cells with the SV40 T-antigen or some other oncogene, expand these cells in culture, and then turn off the oncogene genetically with resultant redifferentiation. Some success with this approach has been reported.

Another interesting approach, reported by Lipes *et al.*, employed insulin expression in the cells of the intermediate lobe of the pituitary in transgenic mice. Remarkably, not only were these cells able to secrete insulin, but they were also resistant to autoimmune destruction by autoimmunity. Intestinal stem cells are of endodermal origin, which makes them attractive targets. Hopes for this pathway are raised by the demonstration that intestinal epithelioid IEC-6 cells can produce and secrete insulin after transfection with pdx1, followed either by transplantation or by treatment with betacellulin. The engineering of GIP-secreting K cells in the intestine to produce insulin is another novel approach. These cells can secrete insulin along with GIP into the blood stream after the oral administration of glucose. Concern must be raised about how this means could produce insulin in a manner that will be precise enough to be useful clinically.

Attempts to Deliver Insulin Via Constitutive Rather Than Regulated Secretion

The essence of this concept is that glucose could be used to induce the promoter of a gene that could drive the synthesis of insulin, followed by constitutive release by a cell such as the hepatocyte. An example of this approach employed use of adeno-associated virus to transduce liver with the L-type pyruvate kinase promoter that responds to glucose, to induce the production of single chain insulin. The advantage of single chain insulin is that it does not need to be cleaved by the convertases normally needed for proinsulin to become biologically active insulin. It was shown that high plasma glucose levels could stimulate increases of plasma insulin that were sufficient to bring glucose levels of diabetic mice into the normal range. Important questions have been raised, however, about whether this approach could ever be clinically useful. A recent perspective article has pointed out that the timing of insulin release by a gene-dependent constitutive secretory mechanism seems highly unlikely to be useful for type 1, or even type 2, diabetes, in which more rapid insulin delivery or suppression will be needed to cope with the normal dynamics of metabolism found with eating, fasting, and exercise.

CONCLUSION ON ADULT SOURCES OF NEW ISLET CELLS

The quest to find insulin-producing cells that might be used for transplantation is intense. Rapid improvements in our understanding of the mechanisms of cellular development and a wide array of potential stem or precursor cell candidates provide fuel for optimism that adult cells could solve.

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KEY WORDS

Beta cell Insulin-producing cell contained within pancreatic islets.

Islet neogenesis Generation of new pancreatic islets from precursor cells.

Islet transplantation Transplantation of isolated pancreatic islets with the intent of curing diabetes.

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Chapter 58

Burns and Skin Ulcers

Edward Upjohn, George Varigos, and Pritinder Kaur

INTRODUCTION

Burns and skin ulcers are major causes of morbidity and, in the case of burns, mortality, in both the developed and developing world. Epidermal cells have been used in the therapy of these conditions for decades in the form of autologous skin grafts. For the last 20 years, advanced cell culture techniques have permitted the development of methods to identify and even isolate viable skin stem cells that could potentially be used in the treatment of these two conditions. These advances provide a strong foundation for building more advanced and exciting therapies based on stem cells to treat these challenging conditions.

The epidermis of the skin is a constantly renewing stratified squamous epithelium. It consists mostly of keratinocytes, but also of Langerhans cells, melanocytes, and Merkel cells resting on a supporting dermis that contains the nerve and vascular networks, which nourish the epidermis. The dermis is also the location of epidermal appendages, fibroblasts, mast cells, macrophages, and lymphocytes. Epidermal stem cells are responsible for the ability of the epidermis to replace itself, both in normal circumstances and in traumatic skin loss, such as from burns and skin ulceration.

BURNS AND SKIN ULCERS: THE PROBLEM

As with many medical conditions, it is difficult to quantify the burden and impact of these two conditions in a meaningful and tangible way. A study estimating the lifetime costs for injuries in the United States in 1985 rated fire and burns as the fourth largest cause of lifetime economic loss, at $3.8 billion. Only motor vehicles, firearms, and poisonings were responsible for greater losses. Burns are also an important cause of injury in the developing world, where more traditional methods of lighting and cooking, such as oil lamps and open fires, are still commonplace. Skin ulcers may be caused by several pathological processes, including infection, trauma, diabetes, and venous ulcer disease. Chronic venous ulceration is a common cause of skin ulcers, with an estimated prevalence of 1–1.3%. Skin ulcers are also difficult and expensive to manage, because of their slow rates of healing and the requirement for expensive and labor-intensive dressing regimes. A cost of care analysis by the Visiting Nurse Association in Boston found the average cost per month in 1992 of an unhealed ulcer to be $1927.89. Diabetic foot ulcers, like venous ulcers, often have a chronic course. In one study, the cost estimates for foot ulcer care over a two-year period in a population of type 1 and type 2 diabetics who developed an ulcer was $27987. These figures, especially when extrapolated to include lost productivity, are almost beyond comprehension. What is easily comprehended, however, is the personal cost and burden borne by patients suffering from burns or skin ulcers.

EPIDERMAL STEM CELLS

The epidermis of the skin is a multilayered, continuously self-renewing tissue, replaced every 30 to 60 days in human skin. *In vivo* cell turnover studies in mice have shown that all proliferative activity is restricted to the basal layer, which generates the mature functional suprabasal keratinocytes. Thus, epidermal stem cells reside within the basal layer, and are characterized by their capacity for self-maintenance and self-renewal. In addition to producing more stem cells, the stem cells generate transient amplifying cells that divide three to five times before producing terminally differentiated keratinocytes *in vivo*. The ability to identify slowly-cycling stem cells *in situ*, visualized as ^{3}H-Tdr or bromodeoxyuridine (BrdU) label-retaining cells, has permitted their localization at specific niches, including the deep rete ridges of the interfollicular epidermis and the bulge region of hair follicles (Figure 58-1). The identification of epidermal stem cells *ex vivo* is a more controversial subject, because there is no unequivocal assay for these cells *in vitro*.

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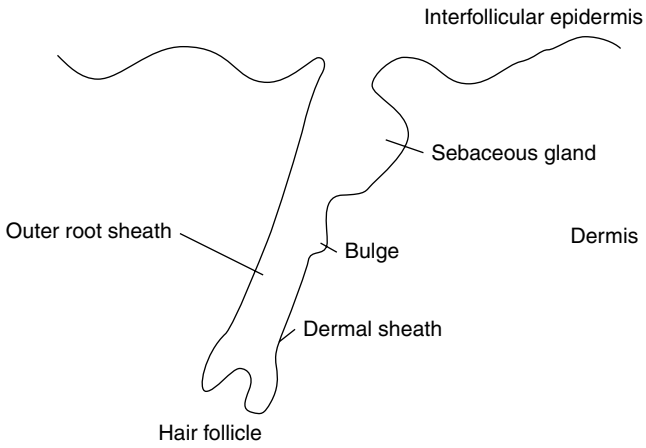


FIGURE 58-1 Epidermis, dermis, and other structures.

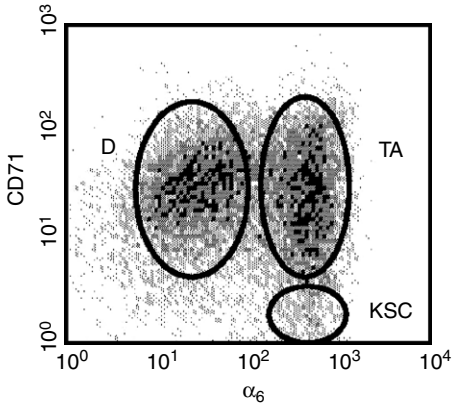


FIGURE 58-2 This FACS plot of primary human, neonatal, foreskin keratinocytes—labeled with antibodies to the cell-surface marker α6 integrin (FITC) and the transferrin receptor (CD71-PE)—reveals phenotypically discrete sub-populations of cells corresponding to stem cells (KSC) with the phenotype α6(bri)CD71(dim), transit-amplifying (TA) cells with α6(bri)CD71(bri), and early differentiating (ED) cells with α6(dim).

Heterogeneity in the growth capacity of keratinocytes was first reported by Barrandon and Green in 1987; they used clonal analysis to identify cells retrospectively capable of generating large colonies of cells exhibiting limited differentiation in culture, termed *holoclones*. These investigators proposed that holoclones were derived from stem cells because of their greater proliferative capacity. This work did not provide a means to prospectively isolate keratinocyte stem cells (KSCs). Subsequent work from Watt and colleagues established that cell-surface markers, specifically β1 integrin, could be used to distinguish basal keratinocytes, with high (β1 bright) or low (β1 dim) proliferative capacity measured in terms of short-term colony-forming efficiency. Despite the initial conclusion that β1 integrin was a marker of KSCs, subsequent work from many laboratories has shown that, although most basal cells are integrin bright, only a small sub-population of these exhibit quiescence, as defined by their ability to retain a ^{3}H-Tdr or BrdU label.

Work from our laboratory has further established that ^{3}H-Tdr label-retaining cells can be purified from integrin-bright keratinocytes on the basis of a second cell-surface marker, i.e., CD71, or the transferrin receptor. Thus, epidermal cells exhibiting the phenotype α6(bri)CD71(dim) represent the stem cell population of both human neonatal and adult murine epidermis. This fraction is enriched for label-retaining cells that are small (~9 μm) with a blast-like morphology, display a high nuclear-to-cytoplasmic ratio, exhibit the greatest long-term proliferative capacity to regenerate keratinocytes *in vitro*, and comprise about 5% of total basal cells (Figure 58-2). Moreover, we have demonstrated that the progeny of KSCs can be distinguished by their cell-surface phenotype: transit-amplifying (TA) cells exhibit high levels of CD71 (α6(bri)CD71(bri)), are enriched for actively-cycling cells, defined as pulse-labeled cells in murine epidermis, and exhibit intermediate keratinocyte cell regeneration capacity; early differentiating (ED) cells are identifiable as α6(dim) cells exhibiting the poorest long-term proliferative capacity and expressing keratin 10 and involucrin—both markers of keratinocyte differentiation (Figure 58-2). This work permits the prospective isolation of KSCs and their immediate progeny by fluorescence-activated cell sorting (FACS), so that the contribution of distinct classes of epidermal progenitors to tissue regeneration during homeostasis or in wound healing can be directly assessed. This work is an important prerequisite to the development of therapeutic strategies using KSCs for skin conditions, including gene therapy. The identification of growth factors that recruit KSCs to proliferate and regenerate tissue will be important to the development of techniques for rapid *ex vivo* expansion that facilitate earlier transplantation for burn victims.

STEM CELLS IN BURNS AND SKIN ULCERS: CURRENT USE

Burns

Autografts

Epidermal cells have been used in the treatment of burns since the introduction of the split skin graft by Karl Thiersh in the late 1800s. Skin grafting to cover defects caused by burns or skin ulceration is limited by the area of skin that may be harvested on any one occasion. Full-thickness grafts (including all of the epidermis and dermis) provide good cosmetic results, but require a primary closure of the donor site, limiting the area that may be grafted. To overcome this problem, the use of split skin grafts was developed, whereby epidermis and underlying dermis is shaved from the donor site to provide a graft. The donor site then re-epithelializes from hair follicles, a

process that is easily seen with the unaided eye and that takes two to three weeks, after which the donor site can be reharvested. It is thought that stem cells of the hair follicle are responsible for healing both the donor site and the grafted area. Experimental evidence in favor of this notion was recently provided by elegant experiments performed by Barrandon and colleagues (2001), who demonstrated that microdissected hair follicle bulges (enriched for stem cells) from transgenic mice expressing the β-galactosidase gene could regenerate interfollicular epidermal tissue, as well as entire hair follicles, when transplanted.

The limitations of skin grafting techniques are the area that can be covered by them, and the many weeks it may take to cover a large area of burn with autologous split skin. Burns of 80–90% are survivable in the short-term with resuscitation, but if coverage of the wounds is delayed because of a lack of grafts, then a high morbidity and mortality is the result.

In the mid-1970s, Rheinwald and Green developed a technique for serial cultivation of epidermal cells, producing a 1000- to 10 000-fold area of graftable epidermis than the initial biopsy (Figure 58-3 and Figure 58-4). These epidermal sheets can then be grafted onto clean wound beds, but they are sensitive to loss by bacterial infection and blistering. In full-thickness burns where the dermis has been lost, the cultured epidermal autograft may be placed directly onto muscle or fascia (Figure 58-5). These cultured epidermal autografts form a permanent covering, suggesting that the stem cells initially cultured and then transplanted have been maintained as stem cells, and therefore retain their crucial role in epidermal maintenance. Histological examination of cultured epithelium reveals the structural similarity to normal epithelium *in vivo* (Figure 58-6). Culturing epidermis is a time- and labor-intensive exercise, estimated to cost between $600 and $13 000 per 1% of the body surface area covered (depending on the proportion of the grafts that successfully take).

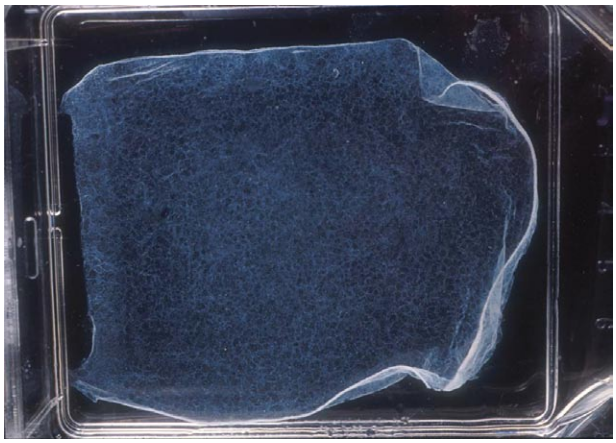


FIGURE 58-3 Detached epithelial graft in a tissue culture flask. Reproduced with the permission of Joanne Paddle-Ledinek.

Allografts

The development of allografts has been driven by the lack of available donor sites for split skin grafting in patients with massive burns, and by the time taken to grow cultured autologous skin from these patients. Burn therapy requires coverage of the burnt areas to prevent secondary sepsis



FIGURE 58-4 Secondary cultures of the epithelium in an incubator. Reproduced with the permission of Joanne Paddle-Ledinek.

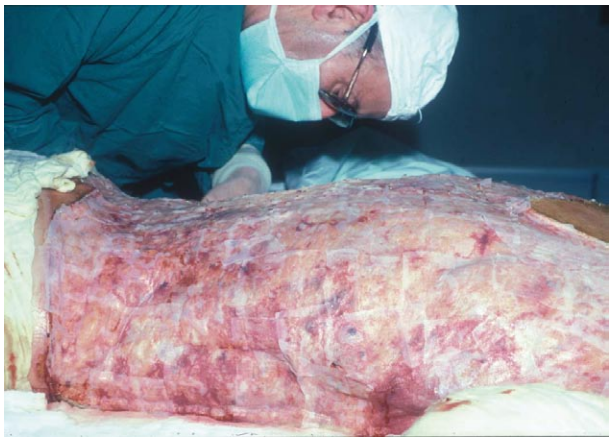
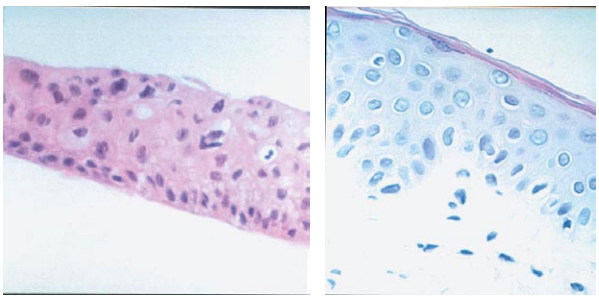


FIGURE 58-5 Application of cultured autograft epidermis to a burns patient. Reproduced with the permission of Joanne Paddle-Ledinek.



(A) (B)

FIGURE 58-6 (A) Side-by-side histological comparison of cultured epidermis and (B) epidermis *in vivo*. Reproduced with the permission of Joanne Paddle-Ledinek.

and other complications. An alternative to split skin grafts is needed that is immediately available, plentiful, effective, and affordable. Cadaveric skin is such an alternative; it is a true allograft and is always eventually rejected by the recipient. As mentioned earlier, skin that lacks a dermis is less able to resist trauma, and is prone to contraction, resulting in a poor functional and cosmetic outcome. Alloderm (Lifecell, Branchburg, New Jersey) is a processed human dermis from which the epidermal and dermal cells have been removed, leaving only the connective tissue matrix. Alloderm can then be applied to burns, and cultured autograft may be placed on top of it. Integra (Integra LifeSciences, Plainsboro, New Jersey) is another dermal substitute developed through the coprecipitation and lyophilization of bovine collagen, chondroitin-6-sulfate, and an artificial epidermal layer of synthetic, polysiloxane polymer. One to two weeks after application, the artificial epidermis is removed, and an ultrathin epidermal autograft (0.003–0.005 inches thick) is placed on the burn area.

All currently available examples of artificial dermis lack a vascular plexus for the nourishment of the epidermis, and require host vasculogenesis into the dermis graft to supply nourishment to the grafted epidermis. Efforts have therefore been focused on encouraging the process of vasculogenesis by genetic engineering of grafts to produce growth factors and cytokines vital to this process (see later sections of this chapter).

Skin Ulcers

Therapy for skin ulcers is based on treating the precipitating and perpetuating factors. This includes antibiotic use for infective ulcers, rigorous pressure care for decubitus ulcers, and compression stockings for venous ulcers (for examples of skin ulcers, see Figures 58-7 and 58-8). The use of occlusive dressing techniques has greatly expanded in recent decades, and these form the foundation for the treatment of many ulcers. In spite of advances such as occlusive dressings, ulcer healing often takes months, if not years, to achieve. In burns therapy, the impetus behind the use of stem cells or cultured epidermal autografts is the need to cover large areas quickly. In ulcer therapy, the time constraints are not as severe, and for definitive closure, split skin grafts remain the gold standard. Cultured skin has been used in the treatment of skin ulcers—in particular, in the use of cultured allografts as a "living dressing." Cultured, autologous outer root sheath cells used in the treatment of chronic decubitus ulcers have been found to produce an "edging effect"—the contraction of the chronic wounds edges in response to the graft, believed to be caused by a release of growth factors, cytokines, and hormones from the outer root sheath cells. Apligraf (Organogenesis, Canton, Massachusetts) is a cultured, bilayered living skin equivalent derived from neonatal foreskin keratinocytes, fibroblasts, and bovine type I collagen. It is indicated for the treatment of venous ulcers and neuropathic diabetic foot ulcers. Chronic wounds (e.g., those with dormant edges) re-epithelialize when exposed to living allograft material. This edge effect, like that seen with outer root sheath cells, is most probably caused by the presence of stimulatory factors. Chronic wounds heal better after repeated application of skin grafts, suggesting that growth factors in the grafts are responsible. Bioengineered tissues, such as Apligraf, probably act as biological systems for delivering growth factors to wounds.



FIGURE 58-7 Leg ulcer. Reproduced with the permission of George Varigos.



FIGURE 58-8 Circumferential leg ulcer healing by granulation. Reproduced with the permission of George Varigos.

FUTURE DEVELOPMENTS

Gene Therapy Approaches in Wound Healing

Epidermal cells can be genetically modified both *in vivo* and *ex vivo*, by both viral and nonviral methods (e.g., recombinant retro and adenovirus infection, liposomes, plasmid injection, and particle bombardment). Initially, gene therapy of epidermal cells was pursued to correct inherited genetic defects, but it is now used to treat wound healing by using genetically engineered keratinocytes as a source of cytokines and growth factors. Fluid

from chronic wounds has been found to be inhibitory to cell proliferation, and to contain degradation products that inhibit keratinocyte migration. The beneficial effect that allografts have on wound healing is thought to be at least partly because of the production of cytokines and growth factors, although the host eventually rejects the allografts. Cultured autografts engineered to produce these cytokines and growth factors in supranormal quantities may expedite wound healing. Epidermal cells can be engineered to express a gene either permanently or transiently. These genes could encode growth factors or cytokines, as mentioned earlier, or they may antagonize some of the inhibitory factors found in chronic wounds.

The vascularization of cultured skin autografts is often delayed when compared to split skin grafting, and may be a contributing factor to the failure of the cultured autograft to take. Cultured skin has been genetically modified *in vitro* to produce vascular endothelial growth factor (VEGF). These modified, cultured skin substitutes have been shown to secrete elevated levels of VEGF, and to have decreased time to vascularization when grafted onto athymic mice.

Tissue Engineering

Tissue engineering of skin is an active area of research and development. Efforts to develop temporary skin substitutes began in the early 1960s. The skin is one of the first organs to have been successfully generated *ex vivo*, and there are several tissue-engineered skin equivalents available today. In bioengineered skin, the epidermal component is either cultured allograft or cultured autograft. The keratinocyte sheets are then combined with the dermal component. The dermal component may be acellular, or it may contain allogeneic or autogenic fibroblasts, or other cells. The optimum skin equivalent would be readily available, could be stored or frozen and ready for use, would be inexpensive to make, would have excellent take, and would give good cosmetic results. Such an ideal skin equivalent does not exist. Split skin grafts fulfill many of these requirements but, unfortunately, they are often not available in a sufficient amount. Currently, the culturing of autografts requires at least three weeks, is labor intensive, and is not available in many developing parts of the world. It would be a great advance if the technique could be made faster and easier to perform. Given our recent ability to use FACS methodology to separate KSC versus TA populations from the epidermis, we have been testing the hypothesis that the stem cell-enriched keratinocytes may provide faster and more reliable skin regeneration in *in vitro* and *in vivo* transplantation model systems. Our recent studies indicate that both epidermal stem cells and TA cells can regenerate epithelial tissue in the short-term fairly rapidly, and that dermal cells, and specific components of the basement membrane (i.e., Laminins), are critical regulators of this tissue-regenerative capacity. Current studies in our laboratory are aimed at understanding how to recruit greater numbers of epidermal stem cells to proliferate *in vitro*, as well as identifying factors capable of promoting stem cell expansion and renewal *ex vivo*. These studies will be important to the development of cellular therapies using epidermal stem cells as vehicles.

SUMMARY

Investigators of epidermal stem cells have the advantage of easy access to their cells of interest. Keratinocytes are also relatively easy to culture and engineer into their normal "organ form" (i.e., sheets of epidermis). As stated earlier, there is an established role for epidermal stem cells in the therapy of human diseases and injuries. The concept of epidermal stem cells, and their application to health care, could form a conceptual framework for the education of the general public about stem cells, and could help to demystify what is commonly perceived to be a complicated science. Epidermal stem cells are excellent targets for gene therapy seeking to correct genetic deficiencies permanently. This could form the basis of new therapies for previously untreatable genodermatoses (such as epidermolysis bullosa), as well as for burns and skin ulcers.

Epidermal appendages such as hair, sweat glands, and sebaceous glands are often destroyed by burns, and their replacement with bioengineered equivalents is still to be achieved. The ability to develop a hair-bearing skin replacement, for example, would address many of the cosmetic problems caused by burns to the face and scalp.

The stem cells of the dermis have yet to be definitively identified and localized within the skin; this can be attributed to the complex cellular heterogeneity of this tissue. Tissue engineering of a replacement dermis to graft onto burns or other defects lacking a dermis would clearly benefit from the incorporation of dermal stem cells. Given that the dermis provides growth factors regulating epidermal growth and morphogenesis, as well as "hair inductive" capacity, further elucidation of its molecular, cellular, and functional components is essential to the development of cellular therapies.

The risk of viral transmission remains a concern with allografts, but they can be frozen, thawed, and used when needed. This is very convenient, and contributes to their lifesaving potential. Tissue engineering with bovine or other sources of collagen must be carefully managed, to reduce the risk of transmission of prion and other diseases to humans. For these reasons, research into cultured autografts will continue, with the goal being a cultured skin equivalent made from epidermal and dermal stem cells, tissue engineered to provide rapid wound coverage with excellent take. The great advances made in the last

quarter of a century have saved many lives, in the case of burns, and made lives worth living again, in the case of skin ulcers. Our expanding understanding of stem cells and their manipulation will build on these advances to enable even better treatment of these conditions in the future.

ACKNOWLEDGMENTS

We wish to thank Joanne Paddle-Ledinek and Heather Cleland.

KEY WORDS

Allograft Cultured allogeneic keratinocyte graft grown from a donor's skin cells, which is eventually rejected by the recipient's immune system (may also have a dermal component).

Autograft Cultured autologous keratinocyte graft grown from the recipient's own skin cells, not rejected by the recipient's immune system.

Epidermal stem cells Slow-cycling and self-renewing cells found in the basal layer of the epidermis responsible for its constant repopulation.

Holoclone A colony of cultured epithelial cells thought to be produced by keratinocyte stem cells.

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Chapter 59

Stem Cells and Heart Disease

Annarosa Leri, Piero Anversa, and Jan Kajstura

THE HEART IS A SELF-RENEWING ORGAN

A fundamental issue concerning the ability of the heart to sustain cardiac diseases of ischemic and non-ischemic origin is whether myocardial regeneration occurs in the adult organ, or whether this growth adaptation is restricted to prenatal life, severely limiting the response of the heart to pathological states. The concept of the heart as a terminally-differentiated organ unable to replace working myocytes has been at the center of cardiovascular research and therapeutic developments for the last 50 years. The accepted view has been that the heart reacts to an increase in workload only by hypertrophy of the existing myocytes during postnatal maturation, adulthood, and senility. When myocardial hypertrophy is exhausted, cell death and heart failure supervene. In contrast to the widespread acceptance of this dogma, there are numerous findings indicating that the mammalian heart has replicative potential, and its response to abnormal elevations in load can be accompanied by myocyte proliferation.

In spite of accumulating results in favor of the formation of new myocytes in a variety of physiological and pathological conditions, the notion of myocyte formation in the adult heart continues to be challenged. It is rather remarkable that cardiologists and cardiovascular scientists are willing to believe that ventricular myocytes in human beings work and live throughout the lifespan of the organ until death of the organism. The possibility of a low turnover of cells as part of cardiac homeostasis is constantly being rejected. Moreover, myocyte death occurs as a function of age, and the progressive loss of cells in the absence of myocyte multiplication would decrease the number of cardiomyocytes to an unrealistic value, incompatible with cardiac function.

Recent findings have demonstrated that the heart belongs to the group of self-renewing organs, such as the hematopoietic system, the intestine, and the skin. The adult rat heart contains a population of undifferentiated cells (Figure 59-1A–C), which express surface antigens typically found in hematopoietic stem cells (HSCs): c-kit, MDR1, and Sca-1. C-kit-positive cardiac progenitor cells (CPCs) have been isolated from the ventricular myocardium, and long-term cultures obtained. They possess the fundamental properties of stem cells: self-renewal, clonogenicity, and multipotentiality. Clonogenic c-kit-positive CPCs differentiate into myocytes, endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblasts (Figure 59-2A–C). Subsets of cardiac cells lacking c-kit, but positive for Sca-1 and ABC transporter, have been described in the mouse heart. These cells also possess epitopes of ECs and HSCs, raising questions about their myocardial origin or specification. The prenatal, early neonatal Isl1-positive cells have been interpreted as a unique category of c-kit-negative Sca-1-negative CPCs. Isl1 and GATA4 are transcriptional co-activators of the myocyte transcription factor MEF2C, indicating that Isl1-positive cardioblasts are committed to the myocyte lineage, and thereby lack the fundamental

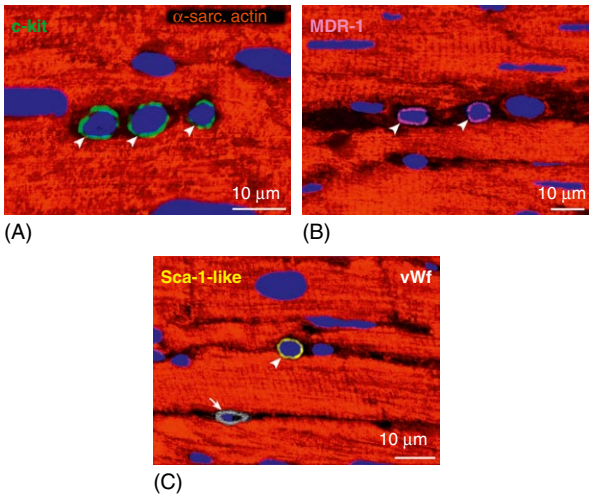


FIGURE 59-1 Sections of adult rat ventricular myocardium showing three c-kit (A: green, arrowheads); two MDR-1 (B: magenta, arrowheads); and one Sca-1-like (C: yellow, arrowhead) positive cells. An endothelial cell expresses von Willebrand factor (C: vWf, white, arrow). Myocytes are identified by α-sarcomeric actin (red).

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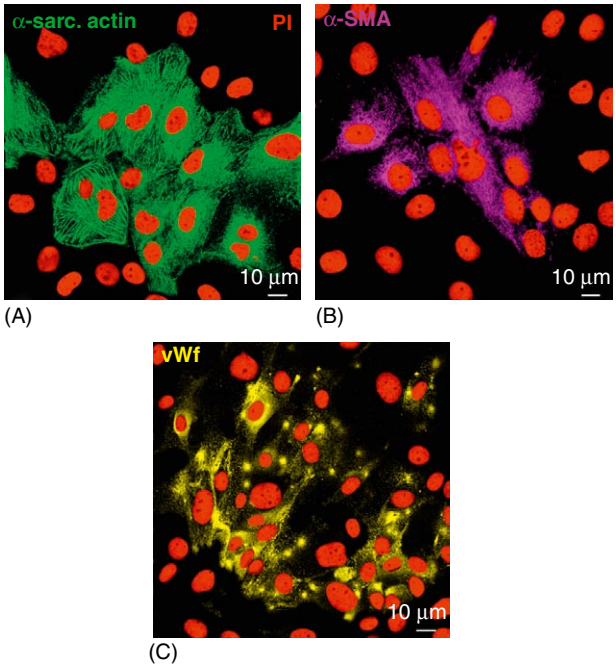


FIGURE 59-2 Differentiation of cardiac progenitor cells *in vitro*. Clonogenic CPCs form myocytes (A: α-sarcomeric actin, green); smooth muscle cells (B: α-smooth muscle actin, magenta); and endothelial cells (C: von Willebrand factor, yellow). Nuclei are labeled by propidium iodide (red).

properties of stem cells. Additionally, Isl1-positive cells are not present in the adult human heart. In contrast, c-kit-positive CPCs do not express markers of endothelial progenitor cells or other bone marrow cells, and are negative for transcription factors and cytoplasmic proteins of cardiomyocytes, ECs and SMCs, including Isl1. Thus, c-kit-positive CPCs show the critical variables required for the classification of stem cells; they are clonogenic, self-renewing, and multipotent *in vitro* and *in vivo*.

As discussed below, two important issues had to be resolved. The first concerned whether c-kit-positive CPCs are present in the human heart, and have implications in myocardial homeostasis and pathology. The second involved the origin of this primitive cell compartment; CPCs may constitute a population of cells which reside in the myocardium during embryonic, fetal, and post-natal development, and throughout the lifespan of the adult organism, or may derive from a circulating pool that continuously migrates from the bone marrow to the myocardium.

THE HUMAN HEART CONTAINS A POOL OF RESIDENT CARDIAC PROGENITOR CELLS

Acute and chronic ischemic heart failure is characterized by myocyte formation, which is mostly limited to the unaffected region of the ventricular wall. Small areas of spontaneous myocardial regeneration which invade the infarcted tissue shortly after the ischemic event have also been identified (Figure 59-3A–C). Primitive cells expressing the receptor of stem cell factor, c-kit, have been detected, raising the possibility that stem cells are present in the normal and pathologic human heart. However, the actual existence of a human cardiac progenitor cell (hCPC) has only recently been shown.

A pool of hCPCs positive for c-kit, but negative for hematopoietic and endothelial cell antigens, has been identified throughout the ventricular and atrial myocardium. The absence of surface epitopes typical of HSCs and endothelial progenitors suggests that hCPCs do not originate from the bone marrow, but constitute a compartment of resident undifferentiated cells. Stem cell niches have been discovered in the cardiac microenvironment, indicating that hCPCs take long-term residence within the heart. Importantly, hCPCs are intimately connected by gap and adherens junctions to myocytes and fibroblasts, which represent the supporting cells within the cardiac niches. Additionally, hCPCs are clonogenic (Figure 59-4A, B) and divide symmetrically and asymmetrically; in the latter case, they give rise to differently-fated daughter cells, a lineage negative stem cell, and an early committed cell. When differentiation is induced, hCPCs acquire the myocyte, SMC and EC lineage.

In vivo, hCPCs create human cardiomyocytes and coronary vessels within the infarcted mouse or rat heart, forming a chimeric organ which provides strong evidence in favor of the critical role that hCPCs have in organ homeostasis and repair (Figure 59-5A–C). Besides their clinical implications, these observations challenge the view of the heart as a post-mitotic organ, and constitute the basis of a new paradigm in which multipotent hCPCs modulate the physiological turnover of the human heart. Understanding the mechanisms of cardiac homeostasis would offer the extraordinary opportunity to potentiate this naturally-occurring process, and promote cardiac repair following injury.

However, the generation of human myocardium within the infarcted mouse and rat heart could have been mediated by different mechanisms:

1. growth and differentiation of hCPCs;
2. fusion of hCPCs with the surviving mouse or rat cardiac cells followed by proliferation of the heterokaryons, and thereby the formation of myocytes, ECs, and SMCs; and
3. a combination of these two processes.

Effective cardiac repair has to occur independently from cell fusion; heterokaryons divide poorly, and have at most a transient positive impact on the age of the fused cells. The evaluation of human, mouse, and rat sex chromosomes (Figure 59-6A–D) has indicated strongly that the generation of human myocardium only involves the

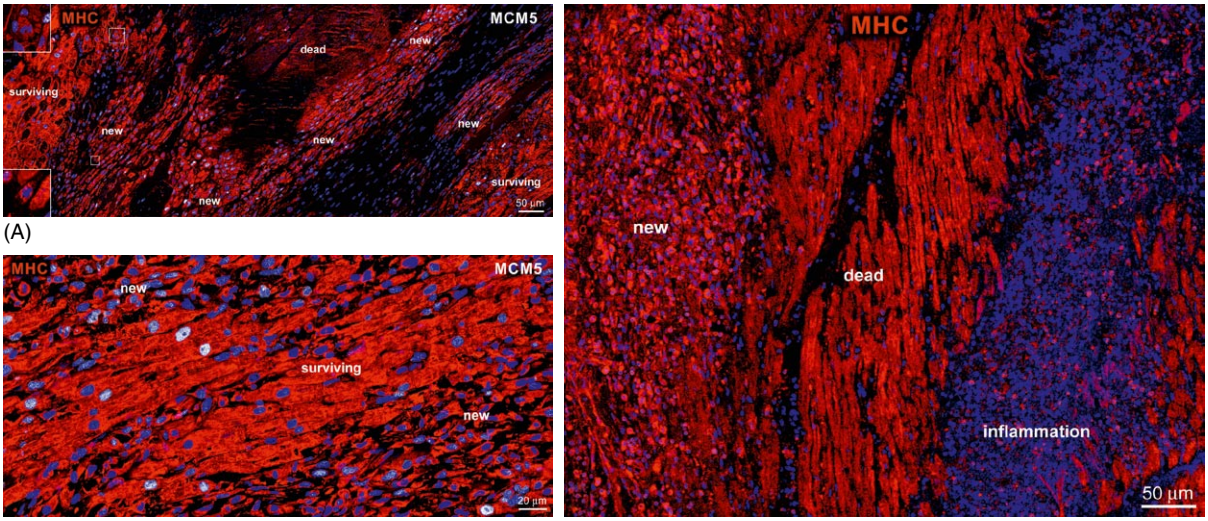


FIGURE 59-3 Areas of regenerating myocardium within the infarcted human heart. Clusters of highly-proliferating small developing myocytes (new) are located in proximity of the necrotic (dead), and spared (surviving) myocardium. Myocytes are labeled by cardiac myosin heavy chain (MHC, red), and nuclei by DAPI (blue). Most of the newly-formed myocytes express the cell cycle protein MCM5 (A, B: white). Two dividing small myocytes are shown in the insets (A). A large infiltrate of inflammatory cells (inflammation) is adjacent to the dead myocardium (C).

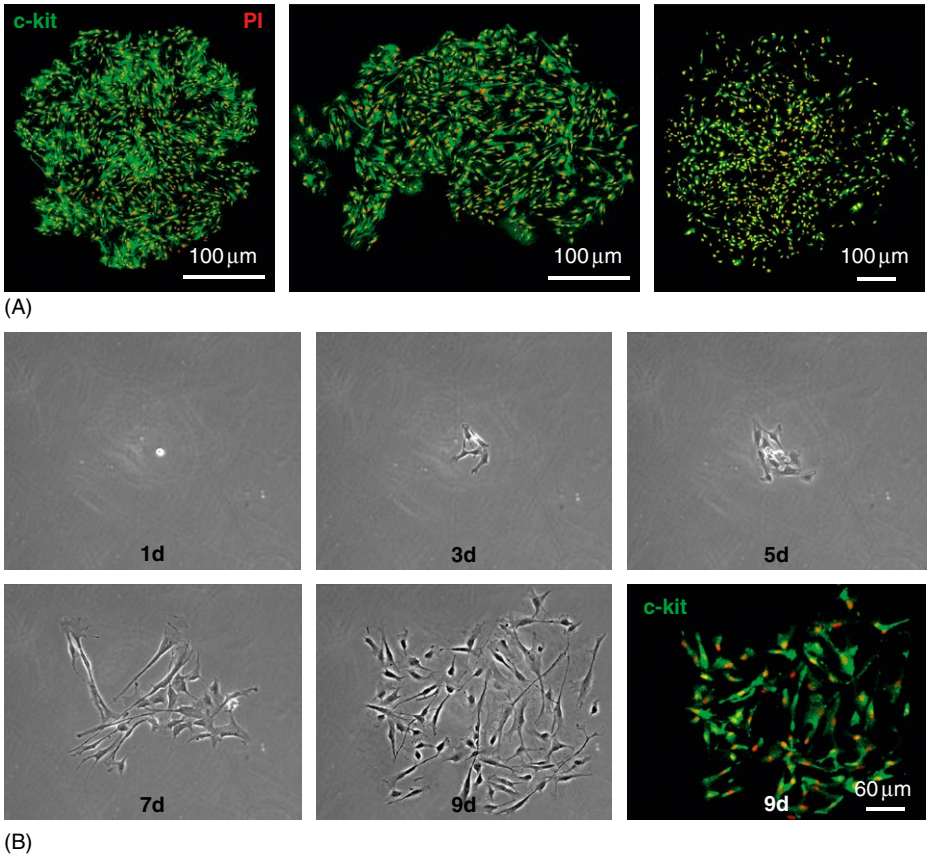


FIGURE 59-4 Clonogenicity of human CPCs. (A) Over a period of two to three weeks, multicellular clones develop from single human CPCs. Cells in the clones continue to express the stem cell antigen c-kit (green). (B) The growth of a clone with time is illustrated by phase-contrast microscopy and confocal microscopy after fixation and staining for c-kit (green). Time in culture (days, d) is shown at the bottom of each micrograph. The number of cells increased with time.

commitment of hCPCs to cardiomyocytes, and SMCs and ECs organized in resistance arterioles and capillary structures. The unlikely involvement of cell fusion was supported by the size of human myocytes, 100–2900 μm^3. If fusion were to be implicated, human myocytes should have a volume of at least 25 000 μm^3 or larger, that is the volume of adult mouse and rat ventricular myocytes. Additionally, it is improbable that fusion of a primitive cell with a terminally-differentiated myocyte induces division of a highly specialized and rapidly contracting cell, permanently

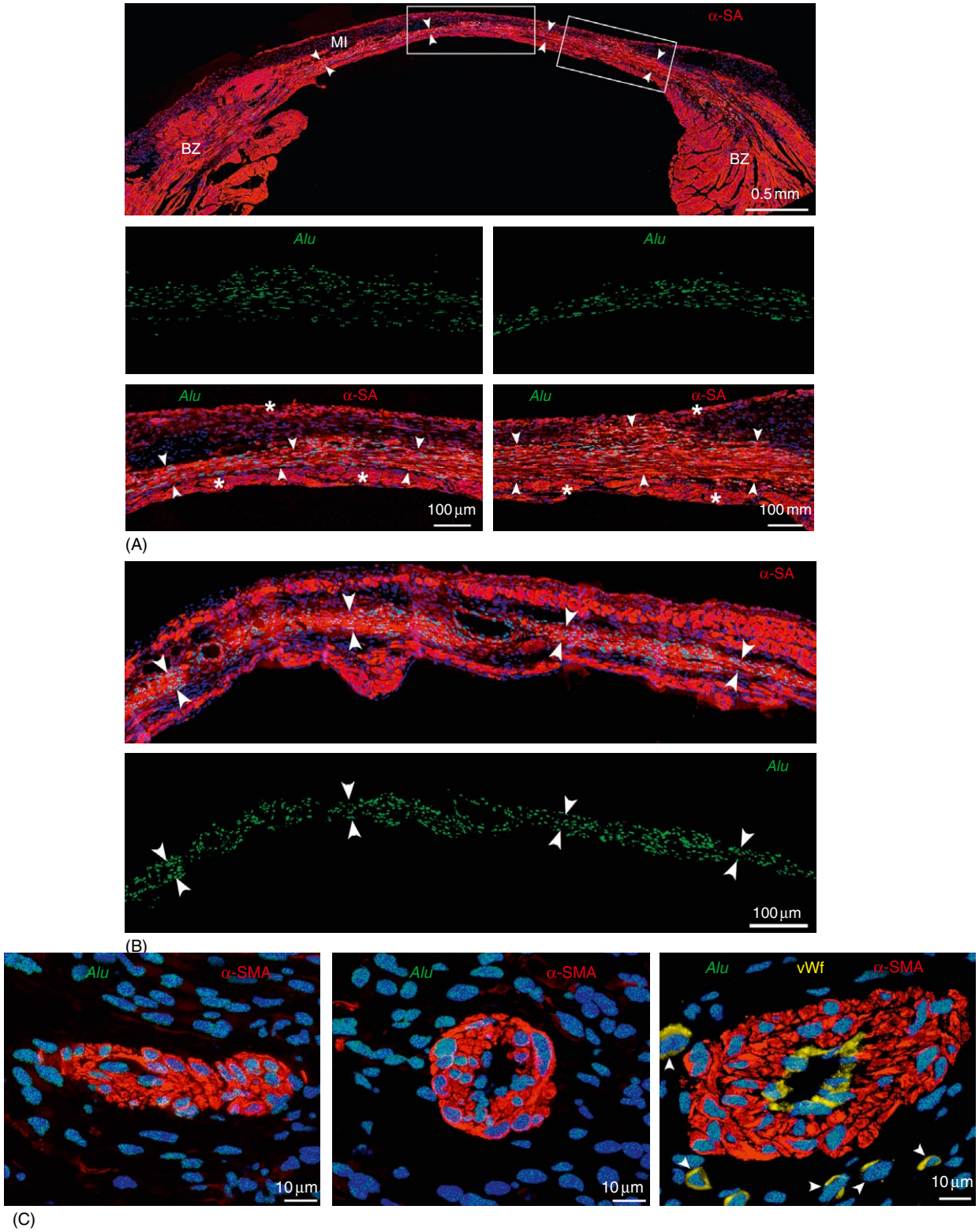


FIGURE 59-5 Human CPCs regenerate the infarcted myocardium. (A) Large transverse section of a mouse heart 21 days after infarction and injection of human CPCs. Human myocardium (arrowheads) is present within the infarct (MI). (BZ, border zone.) Areas in rectangles are shown at higher magnification below. Human myocytes are α-sarcomeric actin (α-SA, red) positive, and contain human-specific *Alu* DNA sequences (green). Asterisks indicate spared myocytes. (B) Large transverse section of a rat heart 14 days after infarction and administration of human CPCs. The band of regenerated human myocytes (upper panel, arrowheads) is positive for *Alu* DNA sequences (lower panel, green). (C) Human CPCs generate coronary arterioles with layers of smooth muscle cells (α-SMA, red) and endothelial cells (vWF, yellow) in the infarcted mouse heart. Capillaries (vWF, yellow; arrowheads) are shown in the lower panel. Cells in the newly-formed vessels are positive for human *Alu* DNA sequences (green).

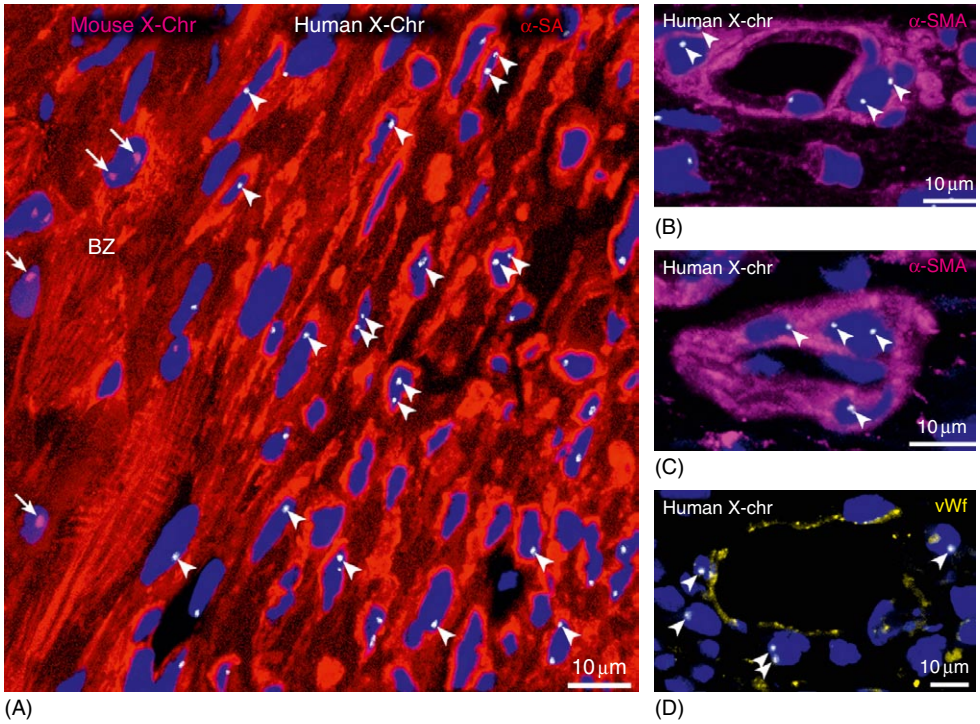


FIGURE 59-6 Cell fusion. Human myocytes (A); and vessels (B–D); show, at most, two human X-chromosomes (X-Chr, white dots, arrowheads). Mouse X-chromosomes (magenta dots, arrows) are present in myocytes of the border zone (BZ). No co-localization of human and mouse chromosomes were found in the newly-formed human cells, excluding that myocardial regeneration depends on cell fusion.

withdrawn from the cell cycle. Similarly, the number of human myocytes formed in the mouse and rat heart markedly exceeded the number of remaining myocytes, further excluding cell fusion.

Since hCPCs promote myocardial regeneration independently of cell fusion, these cells may represent a new form of therapy for the diseased heart. Although CPCs with similar characteristics were shown previously in animal models, the applicability of this information to humans was seriously questioned, and considered a major limitation for the clinical implementation of CPCs. These recent observations support the notion that hCPCs have therapeutic potential; a phase 1 clinical trial has been approved by the FDA.

The identification of a pool of resident hCPCs is apparently at variance with the small foci of myocardial regeneration present after acute and chronic infarcts or aortic stenosis in humans. The limitation that resident hCPCs have in reconstituting myocardium after infarction has been interpreted as the unequivocal documentation of the inability of the adult heart to create cardiomyocytes. The inevitable evolution of ischemic injury is myocardial scarring, with loss of mass and contractile function. A possible explanation of this apparent paradox has been obtained in animal models of the human disease. Stem cells are present throughout the infarcted myocardium but, in spite of the postulated resistance of these cells to death stimuli, they follow the same pathway of cardiomyocytes, and die by apoptosis and necrosis. The fate of hCPCs is comparable to that of the other cells, and myocyte formation by hCPCs is predominantly restricted to the viable portion of the infarcted heart.

It might come as a surprise, but a similar phenomenon occurs in other solid and non-solid organs, including the skin, liver, intestine, and kidney. In all cases, occlusion of a supplying artery leads to scar formation mimicking cardiac pathology. In the presence of polyarteritis nodosa and vasculitis, micro-infarcts develop in the intestine and skin, and resident progenitor cells do not repair the areas of damage. Similarly, infarcts of the bone marrow are seen with sickle cell anemia. Progenitor cells appear to be properly equipped to modulate growth during postnatal development and regulate homeostasis in adulthood. However, progenitor cells do not respond effectively to ischemic injury, or late in life to aging and senescence of the organ and organism.

The recognition of a pool of undifferentiated cells in the adult heart raises a question concerning the origin of this primitive cell population. Whether hCPCs are generated within the myocardium, or are continuously supplied to the heart by the bone marrow through the blood stream and the systemic circulation, cannot be completely resolved. Current knowledge supports the notion that primitive cells are present in the heart at the very beginning of embryonic life, and regulate heart morphogenesis and postnatal development. By introducing the EGFP-gene in the mouse embryo, at the very early stage of the morula-blastocyst transition, the patterns of myocardial histogenesis have

been defined, and the existence of an individual common progenitor of cardiomyocytes in prenatal and postnatal life has been demonstrated. This study has led to the demonstration of the intracardiac origin of adult myocytes, and has excluded the possibility that a secondary immigration of stem cells from distant organs occurs in adulthood under physiological conditions. Homing and engraftment of stem cells through the systemic circulation and the blood vessels would lead to a scattered localization of extracardiac-derived myocytes, with small accumulation in the proximity of capillaries and arterioles. However, single cardiomyocytes or miniclones distributed independently from the original embryonic EGFP-positive patches were never observed. Thus, "late comers" do not contribute to the postnatal growth of the heart and, most likely, progenitor cells from other organs do not have a direct spontaneous role in myocardial homeostasis.

ANATOMICAL DISTRIBUTION OF CARDIAC PROGENITOR CELLS

Differences in the organization of myocyte bundles, and in the level of mechanical forces, are present in the various regions of the heart. The heart resembles a truncated ellipsoid, and because of this peculiar shape, diastolic and systolic wall stresses are higher at the base-mid-portion of the left ventricle and lower at the apex. The atria constitute the cardiac compartment exposed to minimal levels of myocardial stress. These variables may play a critical role in dictating the function and fate of CPCs. This information is crucial for understanding the effects of structural and physical factors on the behavior of adult CPCs. Although stem cells possess strong cellular defense mechanisms, they are sheltered in specialized structures, the niches. Stem cell niches provide a microenvironment designed to preserve the survival and replication potential of stem cells. Primitive cells divide rarely, and those endowed in niches have a much higher probability of self-renewal. For example, the slow-cycling cells of the hair follicle are confined to the bulge where niches are located, where they give rise to the epithelia of the follicle, and contribute to the renewal of the epidermis. This region ensures good physical protection, and is rich in melanin to prevent DNA damage induced by ultraviolet light. Importantly, myocardial niches are distributed preferentially in the atria and apex, which are the anatomical areas most protected by hemodynamic load and wall stress. The recognition that stem cells are clustered in specific regions of the heart exposed to moderate mechanical forces favors the notion that these cells are organ-specific.

Niches are present in all self-renewing organs. The adult heart typically shows discrete ellipsoidal structures in the myocardial interstitium where CPCs are clustered together, in close association with myocytes and fibroblasts. CPCs, myocytes, and fibroblasts are physically connected by adherens junctions expressing N- and E-cadherin, and by gap junctions identified by intercellular calcein dye transfer and the expression of connexin 43 and 45. Because of these properties, myocytes and fibroblasts appear to operate as supporting cells within the cardiac niches. The microenvironment that surrounds the cell populations of the niches consists mainly of fibronectin and α2-laminin (Figure 59-7A–C). Thus, cardiac niches provide the necessary, permissive milieu for the long-term residence, survival, and growth of CPCs. The identification of cardiac niches, and the documentation that CPCs have phenotypical characteristics distinct from HSCs, offers further evidence in support of the notion that the heart is a self-renewing organ regulated by a stem cell compartment.

The localization of stem cell niches is complex in mammalian systems. This search is more readily accomplished in simpler organisms such as *Drosophila*. The majority of information about the cellular components, extracellular proteins, and signaling regulatory pathways of the niches has been obtained in the fruit fly. The precise architectural organization of germ cells in *Drosophila* gonads, and the well-oriented apically-located supporting cells, facilitates the recognition of niche structures. In mammals, the skin niches in the bulge of the hair follicle, the intestinal niches in the crypt, and the neural niches in the subventricular region of the brain have been characterized. Interestingly,

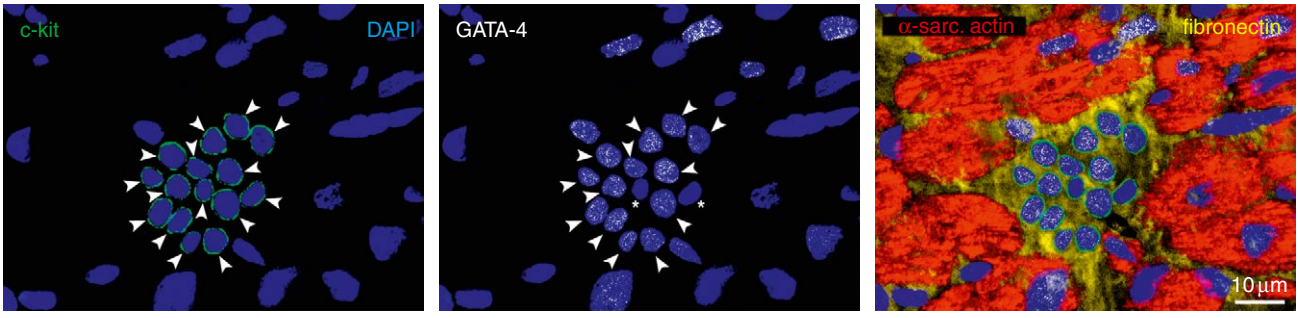


FIGURE 59-7 Atrial niche in the adult rat heart. A cluster of 15 c-kit positive CPCs: (A: green, arrowheads); 13 of which express GATA-4 (B, white). Asterisks indicate two GATA-4 negative cells. Cells in the niche are nested in fibronectin (C, yellow). Myocytes are identified by α-sarcomeric actin (C).

HSCs were identified decades ago, but their niches have only recently been described. The spatial arrangement of CPCs in the cardiac niches is similar to that found in the bone marrow and the brain, providing elements of analogy for these three organs. However, an uninterrupted basement membrane, delimiting the entire pocket of cells, is present in neural and bone marrow niches, but was not recognized in the heart. Cardiac niches are surrounded by bundles of myocytes irregularly oriented.

The undifferentiated state of CPCs appears to be governed by the expression of the α4β1-integrin receptor bound to the α2-chain of laminin and fibronectin. The α1-integrin subunit is highly-expressed on the surface of the interfollicular stem cells of the epidermis, and in neural stem cells, but the α4β1-integrin heterodimer has never been linked to the preservation of stemness in other organs, and may be a unique property of CPCs. The α4β1-integrin receptor is present on erythroid cells, where it drives the formation of mature erythrocytes. Collectively, these observations suggest that in the cardiac niches the α2-chain of laminin and fibronectin transduce mechanical signals from the extracellular compartment to the α4β1-integrin receptor that activates effector pathways opposing CPC commitment and differentiation. This receptor–ligand interaction may have an important role in the homeostasis of cardiac niches.

Homeostasis of the cardiac niches is mediated by asymmetric and symmetric division of CPCs. Asymmetric division is the predominant form of CPC replication in the young mouse heart; it accounts for ~60–70% of proliferating CPCs. The inhomogeneous intracellular segregation of proteins at the time of mitosis constitutes the intrinsic determinant of CPC fate. Numb and α-adaptin interact to enable single primitive cells to produce differently-destined sibling cells. Numb is expressed during mitosis, from late prophase to telophase, and in the early stages of life of the daughter cell. Numb localizes to endocytic vesicles, where it binds to the endocytic protein α-adaptin, promoting the internalization and inactivation of the Notch receptor. Numb-negative cells, however, retain their responsiveness to Notch, and adopt the fate associated with Notch activation. Notch signaling mediates numerous developmental cell decisions, promoting cell differentiation in the prenatal and adult brain. In the hematopoietic system, Notch leads to transcriptional suppression of lineage-specific genes, limiting the number of cells that adopt a committed phenotype.

The prevailing inhomogeneous localization of Numb and α-adaptin in cycling CPCs partly controls the production of a heterogeneous progeny constituted by daughter cells, which follow divergent paths. The BrdU pulse/chase assay conformed to this pattern of asymmetric CPC division. Over time, the number of CPCs confined to the niches remains constant, suggesting that asymmetric kinetics characterizes most of the growth of CPCs in the myocardium. This mechanism of cell renewal has been described as "invariant," and typically occurs in an organ in a steady state that preserves the stem cell pool, and possesses a high degree of growth reserve to respond to physiological and pathological requirements.

The slow rate of proliferation of stem cells allows their identification, as well as the evaluation of their committed progeny. Cells are labeled by a pulse of DNA precursor, which is incorporated in the nuclei of cycling cells. A prolonged chase period results in the dilution of the label in cells that are rapidly replicating, and in its maintenance in cells that are rarely cycling. Cardiac niches harbor a subset of BrdU retaining cells that reflect the class of lineage-negative CPCs (Figure 59-8A–D). This *in vivo* functional assay unmasked the growth characteristics of CPCs, and their preferential localization within cardiac niches.

The heterogeneity in workload on the heart was assumed to reflect a similar heterogeneity in the generation of parenchymal cells in the myocardium. Since differentiated, weakly BrdU-labeled myocytes are the progeny of dividing CPCs, the percentage of BrdU-positive myocytes suggests that nearly 10–19% atrial and ventricular myocytes are formed in two months in the mouse heart, and this significant turnover rate maintains a constant number of atrial and ventricular myocytes. However, myocyte renewal is higher in the atria and apex than at the base-mid region of the left ventricle, and this heterogeneity in myocyte growth is paralleled by a similar heterogeneity in the number of CPCs in the myocardium. These results suggest that cell death and regeneration are enhanced in regions of the heart exposed to low hemodynamic stress. Thus, the workload is either an independent variable of myocyte lifespan, or a dependent variable that extends myocyte survival.

REPAIR OF MYOCARDIAL DAMAGE BY NON-RESIDENT PROGENITOR CELLS

Major discoveries have been made concerning the biology of adult stem cells:

1. They can differentiate into cell lineages distinct from the organ in which they reside, and into cells derived from a different germ layer. These properties were considered to be restricted to embryonic stem cells.
2. Adult stem cells can migrate to sites of injury, repairing damage in various organs. Neural stem cells have been identified in selective regions of the brain that, like the heart, was considered a post-mitotic organ.

Thus, adult stem cells may exist in other unexpected organs, and stem cell behavior is not dictated by the source. HSCs can replace bone marrow and lymphoid organs by migrating to these sites across the vascular endothelium and along stromal pathways. Moreover,

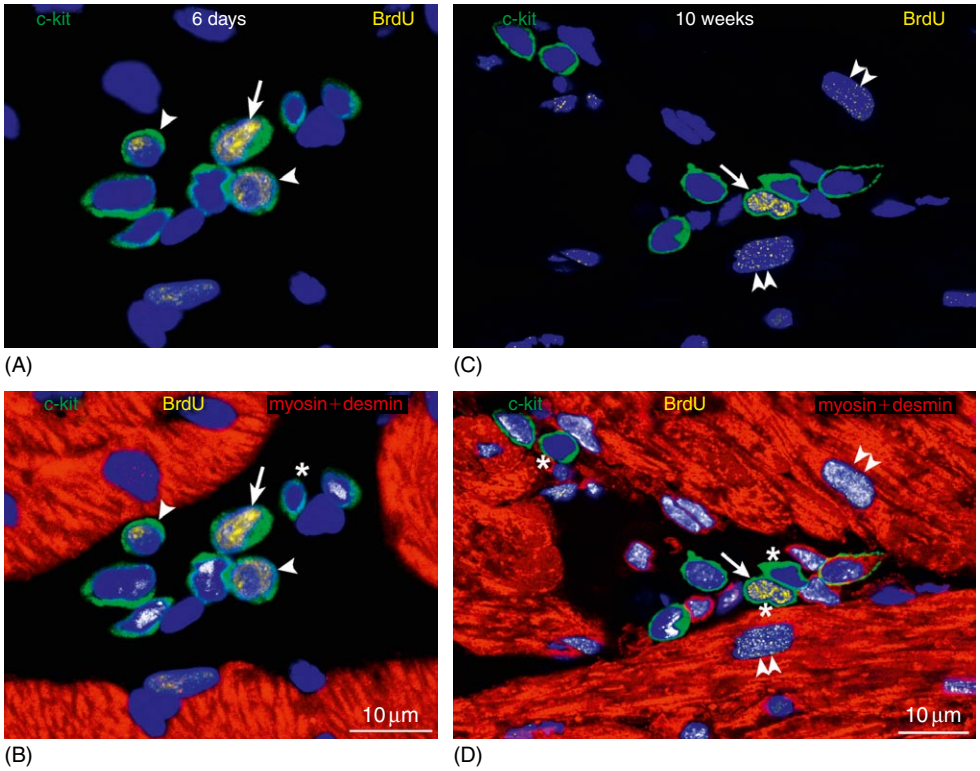


FIGURE 59-8 Long-term label-retaining cells in cardiac niches. To characterize the growth properties of CPCs, 12 injections of BrdU were performed over a period of six days. An additional group of mice was treated in an identical manner for six days, and the animals were sacrificed after a chasing period of 10 weeks. This protocol was employed to identify the pool of long-term label-retaining CPCs. Cells that retain BrdU after the chasing period correspond to stem cells. Sections of atrial myocardium obtained after six days of BrdU pulse (A, B); and after 10 weeks chasing (C, D), were stained for multiple-lineage transcription factors by employing a cocktail of antibodies labeled directly with a fluorochrome. This protocol was utilized to recognize lineage-positive and lineage-negative cells. Atrial niches with bright (arrows) and dim (arrowheads) BrdU-positive (yellow) c-kit-positive-cells (green) are present following six days of labeling (A and B illustrate the same field), and 10 weeks of chasing period (C and D illustrate the same field). Cardiac niches contain c-kit-positive lineage-committed cells (transcription factors, white dots; cardiac myosin and desmin, red) and c-kit-positive lineage-negative cells (B, D; asterisks). Panel D illustrates three lineage-negative cells, one of which is BrdU-bright (arrow) and corresponds to a cardiac stem cell. Two dim-BrdU-labeled-myocytes are also present (C, D; double-arrowheads).

HSCs regenerate skeletal muscle, while skeletal muscle stem cells can repopulate the bone marrow. However, these particular skeletal muscle stem cells are of bone marrow origin. HSCs can also differentiate in functioning hepatocytes. Hematopoietic precursors, injected intravenously, reach the brain where they divide, infiltrate the entire organ and give rise to the cell types of the central nervous system. Conversely, neural stem cells, which derive from the ectoderm, can transdifferentiate into blood cells, which generate from the mesoderm, whereas HSCs can assume the characteristics of neural cells. Together, this information supports the notion that injury to a target organ promotes alternate stem cell differentiation, emphasizing the plasticity of these cells. After an initial wave of enthusiasm, this has become a very controversial issue, and conflicting results have been reported.

In the last few years, efforts have been made to restore function in the infarcted myocardium by transplanting cultured fetal myocytes or tissue, neonatal and adult myocytes, skeletal myoblasts, bone marrow-derived immature cardiomyocytes, and embryonic stem cells. When incorporation of the engrafted cells or tissue is successful, some improvement in ventricular performance occurs. However, these interventions failed to reconstitute healthy myocardium, integrated structurally and functionally with the spared tissue. This defect was particularly evident with skeletal myoblasts, which did not express connexin 43, a surface protein responsible for the formation of ion channels and electrical coupling between cells. Moreover, the vascularization of the implants remained an unresolved issue. These problems pointed to the identification of new therapeutic strategies for the regeneration of dead myocardium. The generalized growth potential and differentiation of adult HSCs administered through the circulation, or locally delivered in areas of injury, suggests that these primitive cells sense signals from the lesion foci, migrating to these sites of damage. Subsequently, homed HSCs proliferate and differentiate, initiating growth processes resulting in the formation of all cellular components of the originally destroyed tissue. Lineage-negative c-kit-positive bone marrow cells implanted in the border zone of an acute infarct translocate to the necrotic region of the left ventricular wall

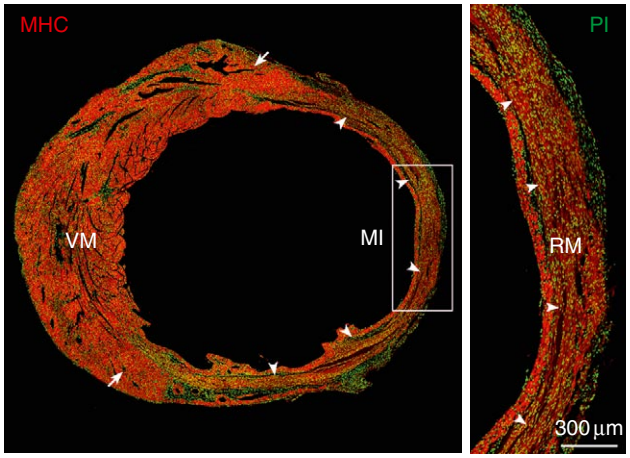


FIGURE 59-9 Bone marrow cells regenerate infarcted myocardium. Transverse section of an extensive myocardial infarct treated with the injection of bone marrow cells in the border zone (arrows). Nine days later, a band of regenerating myocardium was identified (arrowheads). A portion of this band, included in the rectangle, is illustrated at higher magnification in the adjacent panel. Red fluorescence corresponds to cardiac myosin heavy chain labeling of myocyte cytoplasm and green fluorescence reflects propidium iodide (PI) staining of nuclei. VM = viable myocardium, MI = myocardial infarct, RM = regenerating myocardium.

and reconstitute myocardium *de novo*, interfering with scar formation and cardiac decompensation. Numerous small cardiomyocytes and vascular structures develop within the infarct, and partially replace the dead tissue (Figure 59-9). The newly formed young myocardium expresses transcription factors required for myogenic differentiation, cardiac myofibrillar proteins, and connexin 43. Coronary arterioles and capillaries are distributed within the regenerated tissue, and are functionally connected with the primary coronary circulation. Alternatively, cytokines and growth factors may be employed to mobilize stem cells and promote their translocation to damaged organs including the heart. Stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF) are powerful cytokines, which markedly increase the number of circulating bone marrow progenitor cells. These progenitors reconstitute the lymphohematopoietic system of lethally irradiated recipient mice, and in the presence of myocardial infarction home to the heart and follow a differentiation pathway which includes myocytes, vascular Ecs, and SMCs.

These early studies on bone marrow cell (BMC) transdifferentiation have been challenged, and the therapeutic efficacy of this class of progenitors for the infarcted heart has been questioned. The major criticisms include:

1. lack of utilization of genetic markers for the recognition of the donor BMCs and their progeny;
2. inaccurate interpretation of the original data due to autofluorescence artifacts; and
3. the possibility that myocyte regeneration is mediated by fusion of BMCs with resident cardiomyocytes, rather than BMC transdifferentiation.

These important questions of myocardial regeneration have recently been dealt with by utilizing three categories of c-kit-positive BMCs obtained from transgenic mice in which EGFP was driven by the ubiquitous β-actin promoter, or the cardiac specific α-myosin-heavy-chain (α-MHC) promoter; a third category of BMCs was collected from transgenic mice in which a c-Myc-tagged nuclear-targeted Akt transgene was under the control of the α-MHC-promoter. In all cases, male BMCs were injected in wild-type female infarcted mice, so that the destiny of BMCs within the recipient heart was determined by genetic tagging with EGFP, cell fate tracking with α-MHC-EGFP, and α-MHC-c-Myc and cell genotyping by sex-chromosome identification.

By this integrated approach, it was documented that a category of adult cells from the bone marrow shares a degree of developmental plasticity commonly seen in embryonic stem cells; c-kit-positive BMCs engraft in proximity of the infarcted myocardium and differentiate into cells of the cardiogenic lineage, forming functionally-competent cardiomyocytes and vascular structures (Figure 59-10A–D). The regenerated myocardium positively interferes with the development of the postinfarction myopathy, attenuating the dramatic changes in ventricular size and shape, and the progressive deterioration in cardiac function which occur chronically after infarction. Thus, adult BMCs implanted in the infarcted heart lose the hematopoietic fate, and integrate with the host myocardium by establishing temporary niches, which create the microenvironment necessary for the engrafted cells to adopt the cardiac destiny and form *de novo* myocardium.

In the past several years, clinical studies have documented that BMCs administered acutely and chronically after infarction appear to improve ventricular function in animals and humans. Based on these initial observations, double-blind clinical trials have been conducted and, with one exception, the actual efficacy of BMCs in human heart failure has been shown. In spite of positive results, the mechanism by which BMCs improve the outcome of acute myocardial infarction and chronic ischemic cardiomyopathy in humans remains unclear. Currently, efforts are being made to initiate large clinical trials, although uncertainties persist about the actual effects of BMCs on the decompensated heart.

REPAIR OF MYOCARDIAL DAMAGE BY RESIDENT PROGENITOR CELLS

An important question concerns whether BMCs have to be considered the stem cells of choice for cardiac repair, or whether resident CPCs could be selectively mobilized and, ultimately, employed to replace damaged myocardium. CPCs can be expected to be more effective than BMCs in rebuilding dead ventricular tissue. This is because BMCs have to reprogram themselves to give rise to progeny

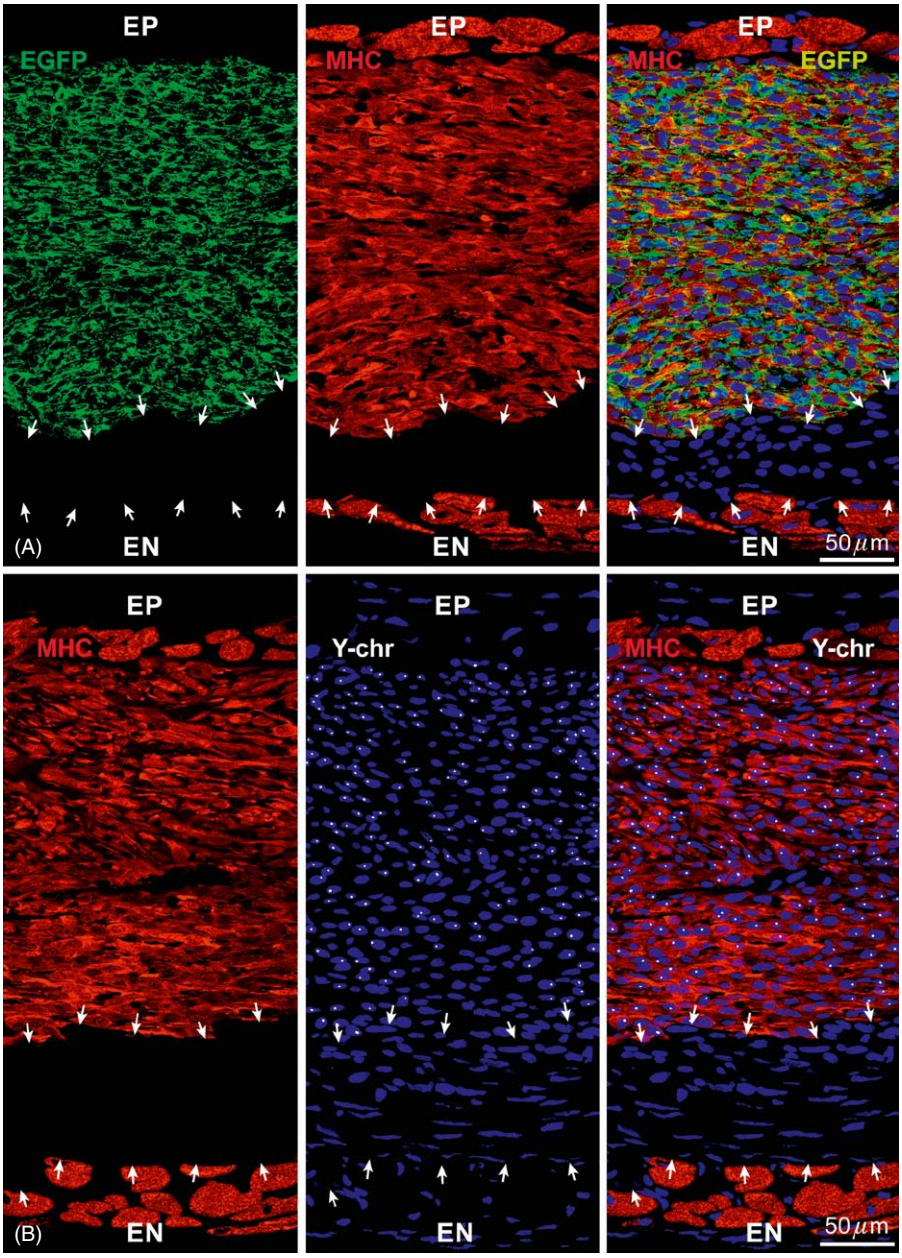


FIGURE 59-10 c-kit-positive bone marrow cells form myocytes and vessels in the infarcted heart. (A, B) Mice treated with bone marrow cells obtained from EGFP-transgenic mice. (A) Left, center, and right panels show EGFP (green), regenerated myocytes (MHC, red), and their merge. Arrows point to non-regenerated infarct. (EP, epicardium; EN, endocardium.) (B) Y-chromosome localization across the infarct. Left, center, and right panels illustrate regenerated myocytes (MHC, red), distribution of Y-chromosome (white dots), and their merge. Arrows point to non-regenerated infarct.

differentiating into cardiac cell lineages. Such an intermediate phase is completely avoided by the direct activation and migration of CPCs to the site of injury. Moreover, CPCs may be capable of reaching functional competence and structural characteristics typical of mature myocytes and coronary vessels in a short time. It is intuitively apparent that the attraction of this concept is its simplicity. Cardiac repair might be accomplished merely by enhancing the normal turnover of myocardial cells. Cell regeneration occurs throughout the lifespan of the organ and organism. This process continuously replaces old dying cells with new better functioning cells. Cell renewal is not restricted to parenchymal cells, but involves all cell populations of the heart. The presence of CPCs has provided a logical explanation for the wide heterogeneity among myocytes in the adult ventricular myocardium. Old hypertrophied myocytes are mixed together with smaller mature cells and cycling amplifying myocytes. Stem cells divide rarely, while committed transit amplifying cells are the actual group of replicating cells in self-renewing organs. The less

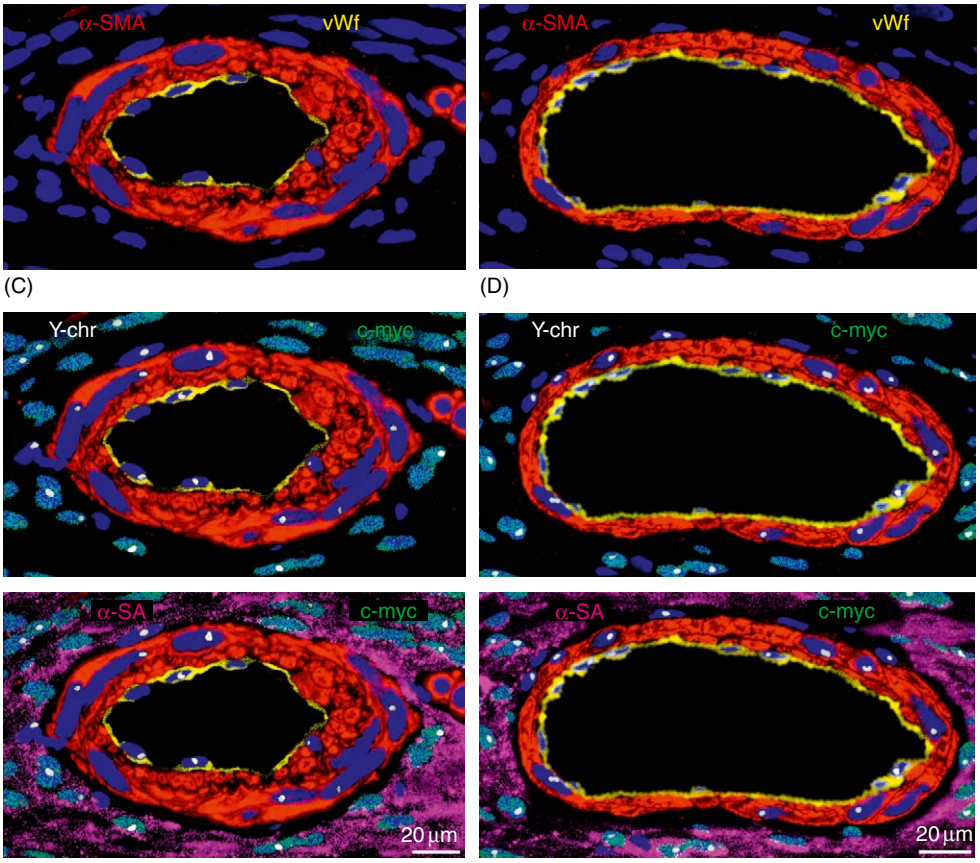


FIGURE 59-10 (C, D) Arterioles formed by bone marrow cells from transgenic mice in which c-Myc-tagged nuclear-targeted Akt was driven by the α-MHC promoter. Smooth muscle cells (α-SMA, red) and endothelial cells (vWF, yellow) carry the Y-chromosome (white dots). The vessel wall is c-Myc-negative, while myocytes (lower panels) are c-Myc-positive (green), carry the Y-chromosome, and are α-sarcomeric actin-positive (α-SA, magenta).

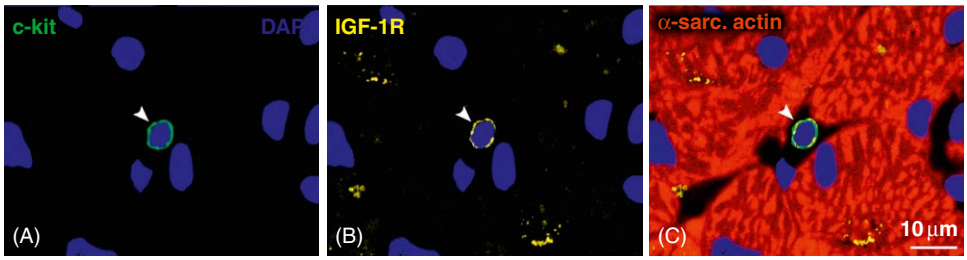


FIGURE 59-11 CPCs express IGF-1R. A c-kit positive cell (A: green fluorescence, arrowhead) expresses IGF-1 receptor (B: yellow, arrowhead). Panel (C) shows the co-localization of c-kit and IGF-1 receptors on the same cell (yellow-green fluorescence, arrowhead). Myocytes are identified by α-sarcomeric actin (red).

primitive amplifying cells possess a unique property; they undergo rounds of doublings and simultaneously differentiate. Thus, CPCs undergo lineage commitment, and myocytes or SMCs and ECs are generated.

CPCs express insulin-like growth factor-1 receptors (IGF-1R) and c-Met, and can be activated by insulin-like growth factor-1 (IGF-1) and mobilized by hepatocyte growth factor (HGF) (Figure 59-11A–C). Based on *in vitro* mobilization and invasion assays, the c-Met-HGF system is responsible for most of the locomotion of these primitive cells. However, the IGF-1-IGF-1R system is implicated in cell replication, differentiation, and survival. These observations have promoted a series of studies in which primitive and progenitor cells have been mobilized from the site of storage in the atria and apex to the infarcted or senescent myocardium in small and large animal models. The reconstituted damaged myocardium is composed of contracting cells and blood supplying coronary vessels, resembling the composition and characteristics of early postnatal myocardial tissue.

As discussed later, the transition from putative CPCs to cardiac progenitors, myocyte progenitors and precursors

and, ultimately, to amplifying myocytes has been seen, in combination with aspects resembling clonogenic growth, in the hypertrophied heart of patients with chronic aortic stenosis. The identification of these forms of growth in humans was critical for the significance of the observations made in animals. The magnitude of growth in the pressure-overloaded left ventricle exceeds the extent of cardiac regeneration detected in rodents, and provides evidence in favor of the ability of the human heart to repair itself. The heart may be viewed as a self-renewing organ, in which the replenishment of its parenchymal and non-parenchymal cells is regulated by a stem cell compartment and by the ability of these primitive cells to self-renew and differentiate. Regeneration conforms to a hierarchical archetype in which slowly-dividing stem cells give rise to highly-proliferating, lineage-restricted progenitor cells. These cells then become committed precursors that, eventually, reach growth arrest and terminal differentiation.

Collectively, a series of reports indicate that resident CPCs can be activated and translocated to damaged portions of the ventricle where, after homing, they initiate an extensive reparative process leading to the reconstitution of functioning myocardium supplied by newly-formed coronary vessels. Theoretically, this approach is superior to the utilization of BMCs for its immediate accessibility to the sites CPC storage. Additionally, CPC differentiation is rapid, and does not require the reprogramming necessary for BMCs to acquire the cardiac cell phenotypes.

A fundamental issue in need of resolution concerns whether CPCs can invade healed infarcts, and grow and differentiate in this hostile environment replacing scarred with functioning myocardium. This possibility would make cell therapy more relevant to the management of ischemic cardiomyopathy and chronic heart failure in humans. Implantation of CPCs in the border of a scarred infarct or the local intramyocardial delivery of HGF and IGF-1 has positive effects on the chronic evolution of ischemic cardiomyopathy. CPCs locally activated by growth factors or injected in proximity of the scar rapidly rescue nearly 45% of the infarct by replacing fibrotic tissue with functionally-competent myocardium (Figure 59-12). Myocardial regeneration protects the infarcted heart from the progressive increase in cavitary dilation, decrease in wall thickness, and deterioration in ventricular function with time.

The invasion of the scarred tissue by CPCs appears to be mediated by enhanced activity of MMP-9, and possibly MMP-14. MMP-9 is critical for the recruitment of bone marrow stem cells and their mobilization from quiescent to proliferative niches; and a similar mechanism may be operative in the translocation to the chronically infarcted heart of growth factor-treated resident CPCs or delivered CPCs. The up-regulation of MMP-9 expression and activity in CPCs is dependent on HGF. Additionally, SDF-1 which is highly expressed in myocytes and ECs after ischemic injury, acts on MMP-9 and promotes the differentiation of CPCs into vascular cells and cardiomyocytes. The lack of increase in MMP-2 activity observed here may favor the stability of SDF-1, which is degraded by this protease.

Importantly, with respect to the intact heart, the content of cytokines and growth factors differs in the scarred myocardium. The enhanced expression of the chemotactic factors sICAM-1, CXCL7, and bFGF in chronic infarcts may create a condition facilitating migration and homing of CPCs. Additionally, the presence of sICAM-1, CXCL7, and TIMP-1 in the scar could have promoted CPC mobilization, EC migration and differentiation, and vessel formation. The increases in CXCL7 and TIMP-1 are restricted to chronic infarcts. The acutely infarcted myocardium displays a cytokine profile that reflects an inflammatory response and tissue reaction to acute injury. However, the higher levels of sICAM-1 and bFGF within the scar are particularly relevant for myocardial regeneration, since

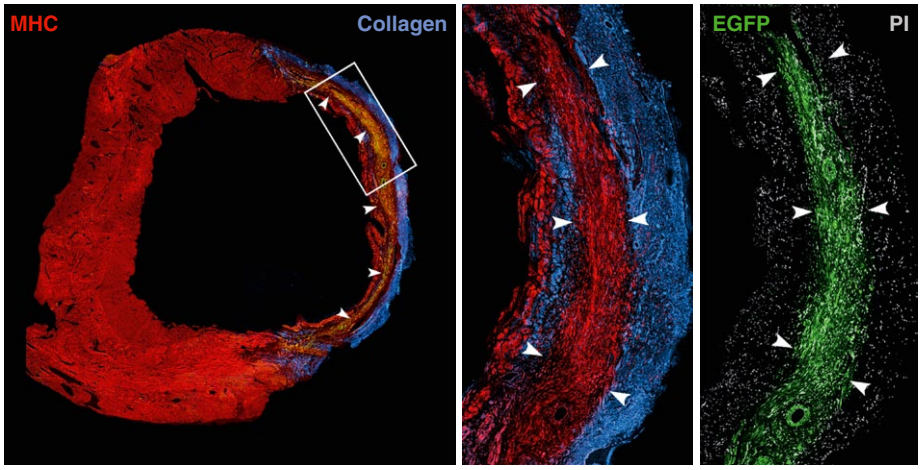


FIGURE 59-12 CPCs regenerate a chronic scarred infarct. A band of regenerated myocytes was present within the infarct (MHC: myosin-heavy-chain, red; arrowheads) following intramyocardial delivery of EGFP-positive CPCs (EGFP, green). The area included in the rectangle is shown at higher magnification in the adjacent panels.

sICAM-1 may create a niche structure that supports the engraftment of CPCs, and bFGF is critical for the differentiation of resident CPCs into myocytes.

The analysis of cytokine and growth factor expression in acute and chronic infarcted hearts offers the possibility to establish whether the delivery of CPCs participate in the synthesis and secretion of soluble proteins which exert an autocrine or paracrine effect on myocardial regeneration. The presence of CPCs in the scar attenuate, at least in part, the differences in cytokine profile with acute infarcts, by increasing the quantity of LIX that is involved in stem cell maintenance and proliferation and the quantity of IP-10 that modulates vessel homeostasis and growth. Thus, acute and chronic myocardial damage lead to the expression of genes that create a microenvironment that conditions the activation, migration, and growth of CPCs, ultimately controlling the regenerative response of the pathologic heart.

Together, the observations in the acutely and chronically infarcted heart suggest that CPCs are a powerful form of cell therapy for the failing heart. CPCs are effective, whether given intramyocardially, via the coronary route, or activated *in situ* with HGF and IGF-1, which trigger their growth and mobilization shortly after an ischemic event and chronically at the completion of healing. CPCs migrate through the myocardial interstitium reaching areas of necrotic and scarred myocardium, where they home, divide, and differentiate into myocytes and vascular structures. From a clinical perspective, CPCs appear to represent an ideal candidate for cardiac repair in patients with acute and chronic heart failure of ischemic and nonischemic origin, in which discrete areas of damage are present in combination with multiple foci of replacement fibrosis across the ventricular wall.

MYOCARDIAL REGENERATION IN HUMANS

According to the dogma, ventricular myocytes in human beings are terminally differentiated cells, and their lifespan corresponds to that of the individual. The number of myocytes attains an adult value a few months after birth, and the same myocytes are believed to contract 70 times per minute throughout life. Because a certain fraction of the population reaches 100 years of age or more, an inevitable consequence of the dogma is that cardiac myocytes are immortal functionally and structurally. This assumption contradicts the concept of cellular aging and programmed cell death, and the logic of a slow turnover of cells with the progression of life in the mammalian heart. Conversely, several reports have provided evidence supporting the notion that myocytes die, and new ones are constantly formed in the normal human heart at all ages. Both processes are markedly enhanced in pathological states, and the imbalance between cell growth (Figure 59-13A, B) and cell death may be an important determinant of the onset of ventricular dysfunction and its evolution to terminal failure and death of the organism. Recent observations have indicated that the human heart contains a population of primitive cells prevalently located in the atria, mimicking the results in rodents. Through growth and differentiation, primitive cells contribute to the remodeling of the stressed heart by generating myocytes, coronary arterioles, and capillary profiles. These newly-formed structures acquire the adult phenotype, are well-integrated in the existing myocardium, and become indistinguishable from the pre-existing tissue components. Such a phenomenon has been carefully documented in cardiac chimerism caused by the migration of primitive cells from the recipient to the grafted heart.

A relevant issue is that the growth potential of the diseased human heart decreases in relation to the duration of the overload, suggesting that primitive cells undergo lineage-commitment, and this process reduces the stem cell pool size. For example, a myocyte mitotic index of nearly 0.08% is detected in the surviving myocardium of the border zone acutely after infarction. However, in postinfarction end-stage failure this parameter becomes 0.015%. Concurrently, myocyte death by apoptosis and necrosis markedly increases, out-setting cell multiplication. Therefore, the question

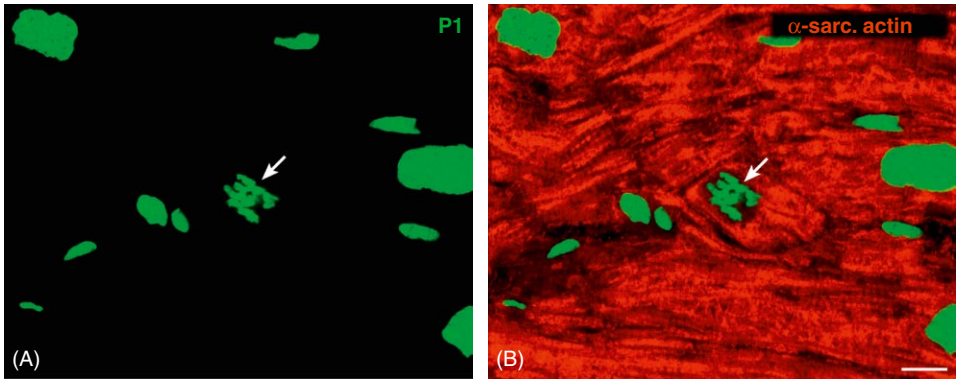


FIGURE 59-13 Dividing myocyte in the left ventricle of a human heart affected by idiopathic dilated cardiomyopathy. Metaphase chromosomes are shown by propidium iodide (A: green, arrow), and the myocyte cytoplasm is stained by α-sarcomeric actin antibody (B: red).

concerns whether interventions can be applied locally to expand the stem cell compartment, or whether this amplification has to be performed outside of the organ in culture systems. The latter is a less favorable strategy, because it requires time and interferes with the urgency of therapy in most patients with advanced cardiac decompensation.

KEY WORDS

Myocardial repair Regeneration of dead myocardium.
Niche Structural and biological entity where stem cells are located, proliferate and differentiate.
Self-renewing organ Organs that possess stem cells.

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Chapter 60

Stem Cells for the Treatment of Muscular Dystrophy: Therapeutic Perspectives

Maurilio Sampaolesi and Giulio Cossu

This chapter deals with the use of stem cells to treat muscular dystrophy. It reviews previous cell therapy trials in mice and patients through the use of donor myoblasts, explaining the likely reasons for their failure, and briefly outlining very recent results on satellite cells that raise new hopes. It then reports the identification of myogenic progenitors in the bone marrow and the related trials, this time in animal models only, to treat dystrophy by bone marrow transplantation. It proceeds to describe recently identified stem/progenitor cells in relationship to their ability to home within a dystrophic muscle, and to differentiate into skeletal muscle cells. Different known features of various stem cells are compared in this perspective, and the few available examples of their use in animal models of dystrophy are reported. On the basis of current knowledge and due to the rapid advance in stem cell biology, a prediction of clinical translation for these cell therapy protocols is outlined.

INTRODUCTION

Muscular dystrophies are caused by progressive degeneration of skeletal muscle fibers (Emery, 2002). Lack of one of several proteins, either at the plasma membrane, or less frequently, within internal membranes, increases the probability of damage during contraction, and eventually leads to fiber degeneration (Blake *et al.* 2002), although the molecular mechanisms are not yet understood in detail. Fiber degeneration is counterbalanced by the regeneration of new fibers at the expense of resident myogenic cells, located underneath the basal lamina and termed "satellite cells." Although satellite cells were considered the only cell endowed with myogenic potential, evidence has accumulated showing that other progenitor cells may participate to muscle regeneration, even though the main player in the process remains the satellite cell (whose pool may eventually be replenished in part by other cell types). These other progenitors are probably derived from distinct anatomic sites, such as the microvascular niche of the bone marrow or skeletal muscle itself, and they reach skeletal muscle either through the circulation or with angiogenesis that accompanies muscle regeneration. Balance between fiber degeneration and fiber regeneration dictates the cellular and clinical outcomes. In the most severe forms, such as Duchenne's muscular dystrophy, regeneration is exhausted and skeletal muscle is progressively replaced by fat and fibrous tissue. This condition leads the patient to progressive weakness and eventual death by respiratory failure (weakness of respiratory muscles), cardiac failure (since dystrophin is also expressed in the heart), or both.

Current therapeutic approaches involve steroids, and result in modest beneficial effects. Novel experimental approaches can be schematically grouped in three major areas (Muntoni and Wells, 2007). The first is gene therapy aiming at the production of new viral vectors (mainly adeno-associated and lentiviral vectors, the latter for cell mediated gene therapy) that are designed to be less antigenic, and more efficient in transducing adult muscle fibers and/or myogenic cells. Second, novel pharmacological approaches focus on high-throughput screens for molecules that may interfere with pathogenetic pathways. The search aims to identify molecules that cause the skipping of termination codons or up-regulate utrophin synthesis, a cognate protein that compensates for dystrophin absence when overexpressed in dystrophic mice; it also includes molecules that simply enhance muscle regeneration or delay protein degradation (Cossu and Sampaolesi, 2007). The final group is cell therapy, based initially on myoblast transplantation, and more recently on the transplantation of stem-progenitor cells (Kuang and Rudnicki, 2007). The recent identification of novel types of stem cells opens new perspectives for cell therapy, which is the topic of this chapter. However, the limited knowledge of stem cell biology is an obstacle that must be overcome to devise protocols with a significant prospect of clinical improvement in patients affected by severe forms of muscular dystrophy.

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MYOBLAST TRANSPLANTATION: REASONS FOR FAILURE AND NEW PERSPECTIVES

This field was opened with a pioneer study by Partridge and his co-workers (Partridge *et al.,* 1989), who showed that intramuscular injection of C2C12 cells, an immortal myogenic cell line derived from adult satellite cells, would reconstitute with high-efficiency dystrophin-positive, apparently normal fibers in dystrophic *mdx* mice. This result caused immediate hopes for therapy that, within a few months, led to several clinical trials in the early 1990s. Myogenic cells were isolated from immune-compatible donors, expanded *in vitro*, and injected in a specific muscle of the patient. These clinical trials failed for several reasons, some of which may have been predicted. (At variance with C2C12, human myogenic cells do not have an unlimited lifespan, and are not syngeneic between donor and host.) Others became apparent long after the start of the trials. For example, most injected cells (up to 99%) succumb first to an inflammatory response, and then to an immune reaction, against donor cell antigens. Cells that survive do not migrate more than a few millimeters from the injection site, indicating that innumerable injections should be performed to provide a homogeneous distribution of donor cells. Even though donor myogenic cells could survive for a decade in the injected muscle (Gussoni *et al.* 2001), the overall efficiency of the process soon made it clear that, although biologically interesting, this approach would have been clinically hopeless.

In the following years, the laboratory of Tremblay focused on these problems, and in a step-wise manner produced a progressive increase in the survival success of injected myoblasts and in their colonization efficiency. Immune suppression and injection of neutralizing antibodies (directed against surface molecules of infiltrating cells such as LFA-1), pretreatment of myoblasts *in vitro* with growth factors, and modification of the muscle-connective tissue all contributed to this improvement. Extension of this protocol to primates has led to a new clinical trial whose initial results have been published (Skuk *et al.*, 2007). However, the fact that this treatment is limited to one muscle, and still requires life long immune suppression, has raised ethical concerns. A possible interesting solution to the problem of immune suppression comes from a recent study where hematopoietic cell transplantation provided an immune-tolerant platform for successful myoblast transplantation in dystrophic dogs (Parker *et al.*, 2008) On a parallel route, several laboratories, including our own, developed strategies to expand *in vitro* myoblasts (and oher cells with myognic potency—see below) from the patients, transduce them with viral vectors encoding the therapeutic gene, and inject them back in at least a few life-essential muscles of the patients from which they were initially derived. This approach would solve the problem of donor cell rejection, but not that of an immune reaction against the vector and the therapeutic gene, a new antigen in genetic diseases. In this case, however, it soon became clear that it was difficult to produce an integrating vector that would accommodate the very large (14-kB) cDNA of dystrophin, whose mutations cause Duchenne muscular dystrophy. The generation of vectors expressing mini- and microdystrophins (containing both protein ends, but missing most of the internal exons) (Harper *et al.*, 2002), or small nuclear RNA (engineered to skip the mutated exon and restore a correct reading frame) (Goyenvalle *et al.*, 2004), appear to have solved at least part of this problem. However, the limited lifespan of myogenic cells isolated from dystrophic patients appeared as an even worse problem. Attempts to solve it, ranging from reversible immortalization with oncogenes or telomerase, to inducible myogenic conversion of nonmyogenic cells, produced interesting results (Zhou *et al.*, 2007) that, however, still require significant work before clinical translation. Moreover, a number of recent studies which increased our knowledge on the biology of satellite cells, showed that expansion in culture, which would be needed both for genetic correction and to reach significant numbers of cells from transplantation, strongly reduced their myogenic potency *in vivo*, so that cultured cells are several-fold less effective in generating donor derived fibers than their uncultured counterparts. Since it is currently inconceivable to directly isolate hundreds of million of cells from a donor biopsy, the reason for this culture-related decrease in myogenic potency will have to be understood and solved before satellite cells may be considered for cell transplantation in patients.

MYOGENIC STEM CELLS IN THE BONE MARROW AND BONE MARROW TRANSPLANTATION

The observation that adult bone marrow contains cells which can participate in muscle regeneration following bone marrow transplantation (BMT) opened a new possible therapeutic option (Ferrari *et al.*, 1998). However, attempts to produce significant dystrophin replacement through this approach failed in dystrophic mice (Gussoni *et al.*, 1999), and later in dystrophic dogs (Dell'Agnola *et al.*, 2004). Even though retrospective analysis in a Duchenne patient who had undergone BMT for other reasons confirmed the persistence of donor-derived skeletal muscle cells over many years, the very low frequency of this phenomenon precluded direct clinical translation for this protocol. Many different studies now indicate that the most likely reason for the low efficiency of muscle regeneration by bone marrow progenitors is, indeed, their low myogenic potency, which may depend on the rare process of cell fusion between a non-myogenic cell and a developing muscle fiber.

The bone marrow hosts well-characterized multipotent cells, such as hematopoietic (HCSs), and mesenchymal cells (MSCs). In addition, a number of different stem-progenitor cells, such as adult progenitor cells (MAPCs), endothelial progenitor cells (EPCs), and mesoangioblasts, have been isolated not only from the bone marrow, but also from other mouse and human tissues, such as the adipose tissue, the dermis, the synovia, and skeletal muscle itself. These cells are described in detail in different chapters of this book; here, we briefly review their their myogenic potential, and consequently their possible use in preclinical models of muscular dystrophy.

Hematopoietic Stem Cells

Until few years ago, no data were available on the ability of purified HSC to differentiate into skeletal muscle. When bone marrow was fractionated into CD45-positive and CD45-negative fractions, the muscle-forming activity after BMT was associated with the CD45+ fraction, suggesting that a myogenic potential is present in the hematopoietic stem cell itself, or in a yet-to-be-identified cell that expresses several markers, in common with true HSCs (McKinney *et al.*, 2002). In the following two years, several reports convincingly demonstrated that bone marrow side population (SP) cells (SP cells, a fraction of bone marrow mononucleated cells that are characterized by dye exclusion) can be recruited to dystrophic or regenerating muscle, and differentiated into skeletal muscle cells on exposure to differentiating muscle cells, or in response to Wnt molecules secreted by recruiting cells (Polesskaya *et al.*, 2003). A fraction of SP cells localizes in a position (between the basal lamina and the sarcolemma) typical of satellite cells, and indeed expresses markers of satellite cells. Moreover, circulating human AC133-positive cells differentiate into skeletal muscles *in vitro* and *in vivo* when injected into dystrophic immunodeficient mice (Torrente *et al.*, 2004).

Two papers subsequently provided conclusive evidence that a single hematopoietic stem cell is able to reconstitute the hematopoietic system of a recipient mouse on BMT, and at the same time give rise to progeny that differentiate into skeletal muscle *in vivo.* The two papers, however, disagree on the mechanism of such a phenomenon: one, in agreement with previous data, identifies donor cells also as *bona fide* "satellite cells" (La Barge *et al.*, 2003); the other failed to identify donor-derived satellite cells, and claimed fusion of a myeloid intermediate progenitor as the cause of differentiation (Camargo *et al.*, 2003). While further work will be required to solve this issue, the presence of a cryptic myogenic potential in true hematopoietic stem cells suggests that it should be possible to import a huge amount of knowledge from both experimental and clinical hematology toward the design of clinical protocols for muscular dystrophy. Still, additional steps would have to be devised, since we already know that BMT is insufficient to ameliorate the dystrophic phenotype. Specifically, homing of progenitor cells to muscle would have to be stimulated since HSC naturally home to the bone marrow; equally important, HSC would have to be directed to a myogenic fate with much higher efficiency than that currently observed. Transient expression of leukocyte adhesion proteins and inducible expression of MyoD may be steps in this direction.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs), originating mainly from pericytes, are located in the perivascular district of the bone marrow stroma, and are the natural precursors of bone, cartilage, and fat, the constituent tissues of the bone. Although MSCs were reported to produce myotubes in culture on induction with 5-azacytidine, they do not differentiate into muscle under normal conditions. When transplanted in sheep fetus *in utero*, human MSCs colonized most tissues, including skeletal muscle, although their effective muscle differentiation was not demonstrated. It was, however, reported that expression of the intracellular Notch domain converted human, cytokine treated, MSC to highly myogenic cells *in vitro* and *in vivo* (Dezawa *et al.*, 2005).

Endothelial Progenitor Cells

Initially identified as CD34+, Flk-1+ circulating cells, endothelial progenitor cells (EPCs) were shown to be transplantable, and to participate actively in angiogenesis in a variety of physiological and pathological conditions. *In vitro* expansion of EPCs is still problematic, and few laboratories have succeeded in optimizing this process. The clear advantage of EPCs would be their natural homing to the site of angiogenesis, which would target them to the site of muscle regeneration. However, their ability to differentiate into skeletal muscle has not been tested.

Multipotent Adult Progenitor Cells

Verfaillie and colleagues recently identified a rare cell within MSC cultures from human or rodent bone marrow: the multipotent adult progenitor cell (MAPC) (Verfaillie, 2005). This cell can be expanded for more than 150 population doublings, and differentiates not only into mesenchymal lineage cells, but also endothelium, neuroectoderm, and endoderm. Similar cells can be selected from mouse muscle and brain, suggesting that they may be associated with the microvascular niche of probably many, if not all, tissues of the mammalian body. Furthermore,

when injected into a blastocyst, MAPCs colonize all the tissues of the embryo, with a frequency comparable to that of ES cells. Because of their apparently unlimited lifespan and multipotency, MAPCs appear to be obvious candidates for many cell replacement therapies, although complete differentiation into the desired cell type still needs to be optimized. For skeletal muscle, neither the frequency at which MAPCs differentiate into skeletal muscle cells after azacytidine treatment, nor attempts to optimize this process have been reported. Recently, however, MAPCs have been shown to restore blood flow and stimulate muscle regeneration in mice with limb ischemia (Aranguren *et al.*, 2008). In addition, the ability of MAPCs to travel through the body using the circulatory route has not been formally demonstrated, although the general features of these cells strongly suggest this to be the case. It will be interesting to see whether MAPCs may restore a normal phenotype in mouse models of muscular dystrophy, and indeed their myogenic potency *in vivo* is being compared with that of mesoangioblasts (see below).

Mesoangioblasts

Mesoangioblasts are vessel associated progenitors, initially isolated from the embryonic mouse dorsal aorta, and later from small vessels of fetal and postnatal muscle in different mammalian species, including humans. Interestingly, embryonic mesoangioblasts express mainly early endothelial markers, while their postnatal counterparts express mainly pericyte antigens. The developmental origin and natural fate of mesoangioblasts is currently under investigation through genetic lineage-labeling experiments.

Inasmuch as mesoangioblasts can be expanded *in vitro* (indefinitely in the case of rodents, and for approximately 20 population doublings in the case of other mammals), are able to circulate and cross the vessel wall in the presence of inflammation, and are easily transduced with lentiviral vectors, they appeared as a potential novel strategy for the cell therapy of genetic diseases. In principle, mesoangioblasts can be derived from the patient, expanded and transduced *in vitro*, and then reintroduced into the arterial circulation, leading to colonization of the downstream tissues. Alternatively, wild-type donor mesoangioblasts can be used, with no need for gene correction, but requiring immune suppression. When both wild-type or dystrophic, genetically corrected, mesoangioblasts were delivered intra-arterially to dystrophic muscle of a-sarcoglycan KO mice (a model for limb girdle muscular dystrophy), they resulted in a dramatic functional amelioration of the dystrophic phenotype (Sampaolesi *et al.*, 2003). More recently, intra-arterial delivery of wt mesoangioblasts, non-DLA matched to GRMD dystrophic dogs, resulted in a partial recovery of muscle morphology and function, dystrophin expression (Figure 60-1), and clinical amelioration. Delivery of autologous mesoangioblasts, genetically-corrected to express human micro-dystrophin, did not result in a similar amelioration, despite widespread expression of micro-dystrophin (Sampaolesi *et al.*, 2006). Based on these results, a first clinical trial with donor mesoangioblasts recently characterized in human muscle (Dellavalle *et al.*, 2007) is planned for the near future.

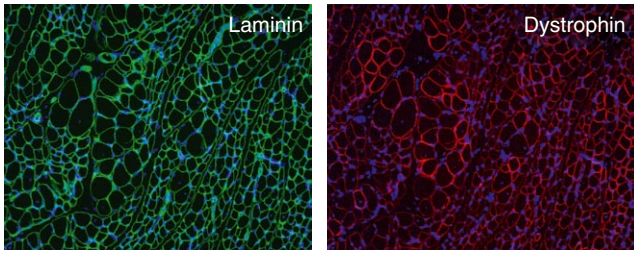


FIGURE 60-1 Expression of dystrophin in Golden Retriever dystrophic dogs after intra-arterial delivery of wild-type mesoangioblasts. Laminin staining (green in the upper panel) identifies muscle fibers in a transverse section of the tibialis anterior of a dystrophic dog, previously injected with 50 million donor cells under a regimen of immune suppression. The majority of these fibers also express dystrophin (red in lower panel), indicating fusion and myogenic differentiation of wild-type donor cells. Nuclei are stained in blue by DAPI.

In conclusion, of the many types of stem cells with a myogenic potential (Table 60-1), only mesoangioblasts have so far been shown to be effective in restoring, to a significant extent, the dystrophic phenotype in animal models of muscular dystrophy.

Myogenic Stem Cells from Embryonic Stem Cells and Inducible Pluripotent Stem Cells

Although embryonic stem (ES) cells hold tremendous promise for future cell therapy protocols, their ability to give rise to skeletal myogenic cells has not been investigated extensively in the past. Recently, however, two groups reported methods to induce efficient skeletal muscle differentiation in human ES, which would robustly produce muscle fibers on transplantation in immune deficient mice in the absence of tumor formation (Barberi *et al.*, 2007; Darabi *et al.*, 2008). In this perspective, the recent creation of inducible pluripotent stem cells (iPS) by forced expression of four genes into adult cells (Yamanaka, 2007) adds a new dimension to this perspective: in fact, the possibility of deriving unlimited number of autologous ES, to be first genetically corrected, and later induced into skeletal myoblasts, would solve the problem of the limited availability of autologous myogenic cells from patients. Even though it is conceivable that this goal will be reached rapidly, and the remaining problems related to iPS (i.e., tumor formation due to cMyc) solved, it should be kept in mind that deriving *bona fide* myogenic cells from iPS will not solve the problem of cell distribution on transplantation that is currently one of the main drawbacks of this strategy (see above).

TABLE 60-1 Potential of Several Types of Mesoderm Stem/Progenitor Cells to Contribute to Muscle Regeneration in a Dystrophic Muscle[a]

Stem Cell	Source	Growth *in vitro*	Crossing muscle endothelium	*In vitro* myogenic differentiation	Contribution to fiber regeneration
SC	Skeletal muscle	Yes	No	High	High but local
HSC	Bone marrow	Modest	Upon induction	Low, co-culture	Modest
MSC	Bone marrow	Yes	Unknown	Upon induction	Modest
EPC	Microvessels	No	Unknown	Unknown	Unknown
MAPC	Bone marrow	Yes	Unknown	Upon induction	Modest
MAB	Microvessels	Yes	Upon induction	Upon induction	Medium-high

[a] A schematic and simplified overview of the features known, presumed, or under scrutiny, of different adult stem cells in the perspective of use for cell therapy (SC: satellite cells; HSC: hematopoietic stem cell, MSC: mesenchymal stem cell, EPC: endothelial progenitor cell, MAPC: multipotent adult progenitor cell, MAB: mesoangioblast). Source is generic, since the exact location within bone marrow is uncertain for some stem cells.

CELLULAR ENVIRONMENT OF A DYSTROPHIC MUSCLE: RESIDENT AND MIGRATORY CELLS

Before the appearance of stem cells on the stage, the histology of a dystrophic muscle could be described with relatively few players. Muscle fibers undergo degeneration for the lack of one important protein, as described previously. This leads to activation of resident satellite cells that proliferate and produce myogenic cells that repair damaged fibers or replace dead fibers with new ones. Satellite cells should also generate new satellite cells (although this has not yet been formally demonstrated) to ensure a reserve population for further cycles of regeneration; failure of this process, especially in the most severe forms of dystrophy, is one major cause of anatomical loss of muscle tissue and functional impairment. However, recent data suggest that even *bona fide* satellite cells (i.e., residing underneath the basal lamina) may be heterogeneous, and contain a sub-population of cells that do not express myogenic markers, such as CD34, Myf5, or M-cadherin, but rather express stem cell markers such as CXCR4, and thus represent higher progenitors, with more robust myogenic potency *in vivo* (Cerletti *et al.*, 2008).

Dystrophic muscle is also infiltrated by inflammatory cells, initially lymphocytes and macrophages, followed by fibroblasts that deposit large amounts of collagen, contributing to progressive sclerosis of the muscle, and adipocytes that replace muscle with fat tissue. The local microcirculation is progressively lost in this process, leading to a hypoxic condition for surviving or regenerated fibers that activates a vicious circle, increasing the chance of further degeneration (Emery, 2002).

A series of studies, which followed the original observation of muscle regeneration by bone marrow-derived cells (Ferrari *et al.*, 1998), demonstrated that additional types

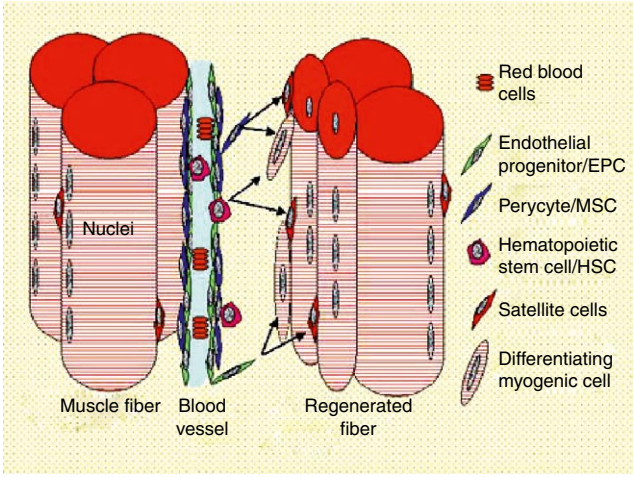


FIGURE 60-2 Possible origin of myogenic cells in regenerating skeletal muscle. Regenerated fibers (characterized by centrally-located nuclei) are formed by fusion of resident satellite cells that may derive, at least partly, from cells derived from the microvasculature as circulating cells (HSCs), endothelial cells (EPCs), or pericytes (MSCs) of the vessel wall.

of progenitor cells are present in muscle. These cells may either be resident in the tissue since fetal histogenesis or blood/vessel-derived, and penetrated into the tissue during the postnatal growth and/or regeneration that occur during normal life, which is much more frequent in muscle diseases. These studies suggested, although not conclusively, a lineage relationship between cells from the bone marrow, or more generally the vascular wall, and resident satellite cells. In the course of this progression, non-myogenic cells may respond to local and perhaps systemic signals, and cross the vessel layers, using a mechanism analogous to leukocytes, to localize initially in a perivascular interstitial district. From there, they may be directly recruited to a muscle-forming population, or may replenish the pool of satellite cells (Figure 60-2). Acquiring a myogenic identity should imply simultaneous loss of other

mesoderm fates, as suggested by the work discussed previously. The existence of mesoderm progenitors in adult muscle which express either endothelial or pericyte markers and can participate to myogenesis supports this possibility. Since this process probably occurs continuously during life, it is possible that any resident satellite cell may derive from the somite-derived myogenic population during embryogenesis or, as we hypothesized previously, from incoming cells that may have followed a circulatory route or may have reached developing muscle with the incoming vasculature during fetal or postnatal, regeneration-triggered angiogenesis. The recent demonstration that virtually all satellite originate from somites (Gros *et al.*, 2005) is not in contradiction of this possibility, as there is ample evidence that endothelial and smooth muscle precursors also originate from somites (Esner *et al.*, 2007; Ben-Yair and Kalcheim, 2008). In such a case, these progenitors may play a major role in tissue histogenesis, but a minor one in adult tissue homeostasis and repair, as would be predictable for a stable, low turnover tissue. This would explain the low numbers of differentiated muscle cells derived from bone marrow or other tissue, and the previous failure of BMT in animal models. On the other hand, the case of mesoangioblasts, which after *in vitro* expansion and genetic correction can be delivered in large numbers to the microvasculature of dystrophic muscle, strongly suggests that this route may overcome a somehow natural limit of these cells and lead to a successful cell therapy.

FUTURE PERSPECTIVES

To be successful, a future cell therapy for muscular dystrophy should probably use stem cells derived from either the patient (autologous) or a healthy, immunocompatible donor. In the first case, cells should meet as many of the following criteria as possible: (1) an accessible source (e.g., blood, bone marrow, fat aspirate, muscle, or skin biopsy); (2) the ability to be separated from a heterogeneous population on the basis of antigen expression; (3) the potential to grow *in vitro* for extended periods without loss of differentiation potency (since it appears unlikely that cells may be acutely isolated in numbers sufficient for therapeutic purposes); (4) a susceptibility to *in vitro* transduction with vectors encoding therapeutic genes (these vectors should themselves meet criteria of efficiency, safety, and long-term expression); (5) the ability to reach the sites of muscle degeneration–regeneration through a systemic route, and in response to cytokines released by dystrophic muscle; (6) the ability to differentiate *in situ* into new muscle fibers with high efficiency, and to produce physiologically normal muscle cells; and (7) the ability to be ignored by the immune system, despite the presence of a new protein (the product of the therapeutic gene) and possibly of some residual antigen from the viral vector.

In the case of donor stem cells from an immunocompatible donor, some of the criteria would be less stringent and some more rigorous. For example, the source of the cells would not need to be easily accessible, since cells from aborted fetal material also may be obtained from sites, such as the central nervous system, not easily accessible in a patient. Gene transduction would not be necessary, since donor stem cells would possess a normal copy of the mutated gene responsible for muscular dystrophy. On the other hand, immune suppression would become a major issue to prevent the rejection of a classic allograft. Although the vast experience accumulated in BMT may be essential to devise appropriate protocols, it should be remembered that some stem cells, such as mesoangioblasts, do not express class II MHC, making control of the immune response less complex than in the case of HSC transplantation.

Through the years and the work of several laboratories, we are now getting close to meeting most, if not all, of the required criteria. Although many steps remain to be completed, it is becoming apparent that there is probably enough information for starting a few phase 1 pilot trials, also considering that the risk of such a new therapy would be justified for a terrible, devastating, and slowly killing disease for which no real therapy still exists, more than 20 years after cloning of the responsible gene. The positive results obtained in dystrophic dogs, despite some erroneous and disingenuous criticism which followed the publication of the article, represent to date the most solid evidence in favor of a possible similar positive outcome in patients.

Hopefully, in very few years, phase 1 clinical trials with stem cells may start, and may set the stage for another, and at least partly successful, attack to defeat these genetic diseases.

ACKNOWLEDGMENTS

We thank G. Ferrari, M. Goodell, J. Huard, F. Mavilio, and M. Rudnicki for critical reading of this chapter and helpful suggestions. We are especially grateful to T. Partridge for thoughtful and provocative insights.

KEY WORDS

C2C12 Immortal myogenic cell line derived from adult murine satellite cells.
ES Embryonic stem cells.
IPS Induced pluripotent stem cells.
MAPCs Multipotent adult progenitor cells.
MCS Mesenchymal stem cell.
Mesoangioblasts (MABS) Vessel-associated stem cells.
Satellite cells (**SC**) Resident myogenic progenitors of postnatal muscle.

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Chapter 61

Regeneration of Epidermis from Adult Keratinocyte Stem Cells

Ariane Rochat and Yann Barrandon

Keratinocyte stem cells, along with hematopoietic stem cells, have the longest record in cell therapy. Following the seminal work by Green and colleagues in the early 1980s, hundreds of burned patients worldwide had their lives saved by the transplantation of cultured autologous keratinocyte stem cells. Reconstitution of a functional epidermal barrier, self-renewal of the epidermis, and the unexpected regeneration of a papillary dermis are major accomplishments. Gene therapy of hereditary, disabling skin diseases using genetically modified keratinocyte stem cells is down the road. Nevertheless, many more challenges lie ahead. The functionality, the mechanical properties and the aesthetic of the regenerated skin must improve. Sweat glands and hair follicles must be reconstructed, and the pigmentation of the skin better-controlled. It is then necessary to thoroughly comprehend the cellular and molecular mechanisms involved in skin morphogenesis, epidermal renewal, stem cell interactions, dermal remodeling, and fetal wound healing. Only then will stem cell therapy become a major therapeutic option in plastic and reconstructive surgery. Nevertheless, several recent developments are extremely promising, such as *ex vivo* keratinocyte stem cell gene therapy, the capacity to derive adult-like keratinocyte from embryonic stem cells, and the capacity to derive induced pluripotent stem cells from adult keratinocytes.

INTRODUCTION

The skin of vertebrates is covered with scales, feathers, or hairs, whose main function is to protect the body from environmental hazards; the skin also contributes to protection, tactile sensation, thermoregulation, and camouflage. Furthermore, it participates in body homeostasis, for example, through water balance in the frog, or vitamin D synthesis in the human. Most importantly, the appearance of the skin shapes emotional and social attitudes, which is best illustrated in the human by the incessant advertising campaigns promoting a young and smooth skin, and by the distress of patients with disabling skin diseases or scars.

The human skin represents about 16% of the body weight and an area of 1.7 m^2 in the adult. It is composed of three layers that are, from the outer to the inner surface, the epidermis, the dermis, and the subcutis. The epidermis is a stratified, keratinized epithelium in continuity with the epithelial part of the skin appendages (i.e., the hair follicles, the sebaceous glands, and the sweat glands (Figure 61-1). The epidermis contains several specialized cell types that contribute to body protection. The keratinocytes, which represent 90% of the epidermal cells, are of ectodermal origin and are responsible for epidermal renewal, cohesion, and barrier function. The melanocytes are pigment-forming cells of neural crest origin, and are responsible for the color of the skin and UV protection, whereas the Langerhans and epidermal T-cells are of marrow origin, and are involved in immunological defense. The nonvascularized epidermis and epidermal appendages are separated from the highly vascularized dermis by a complex basement membrane, which the nutrients originating from the dermal vessels cross. The dermis is a connective tissue that contains fibroblasts producing proteoglycans, collagen, and elastic fibers, and it is responsible for the mechanical properties of the skin. The subcutis, that mainly contains adipocytes (fat cells), is involved in thermoregulation and lipid storage. The skin is also highly innervated, with numerous free nerve endings in the epidermis and specialized nerves endings in the dermis. The function of the neuroepithelial Merkel cells, which rest in the basal layer of the epidermis, is largely unknown. Altogether, the skin contains more than 25 specialized cell types, originating from at least six different stem cells. Most importantly, the skin is constantly renewed and remodeled. In the human, the epithelial part of the epidermis is renewed once every three weeks, and hair follicles undergo regular cycles of growth, regression, and rest about 25 times in a lifetime. Similarly, the dermis is constantly remodeled with new collagen fibers being produced, as old ones are degraded.

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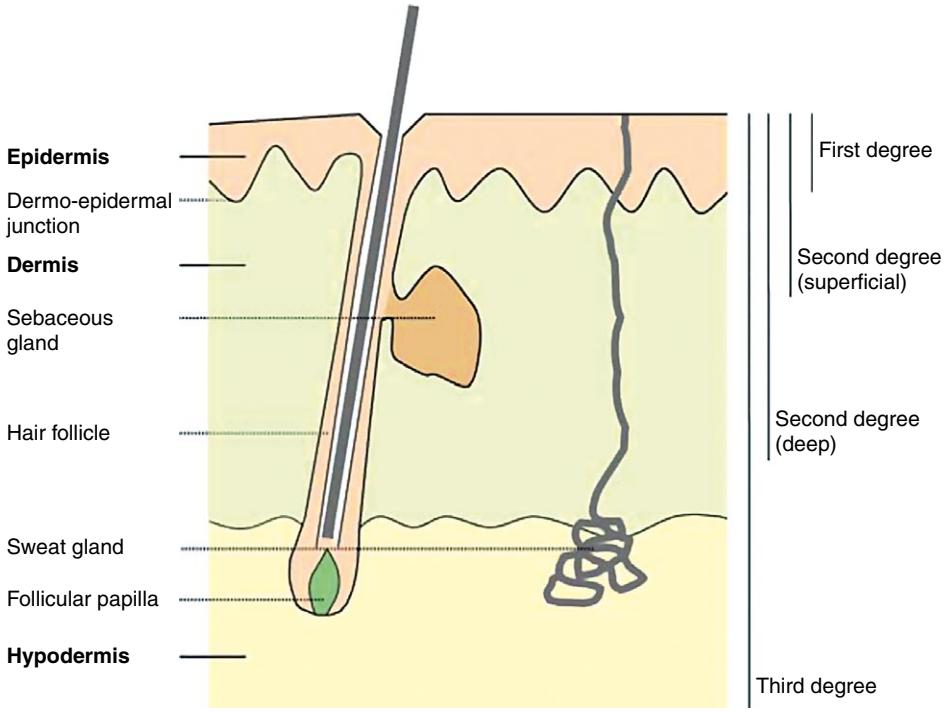


FIGURE 61-1 Depth of burns. First-degree burns heal spontaneously without a scar, second-degree burns heal with a scar. Third-degree burns do not heal, and may necessitate the transplantation of autologous split-thickness skin grafts and autologous cultured keratinocyte stem cells, depending on the extent of the burns.

The Keratinocyte Stem Cell

The keratinocyte stem cell is one of the adult stem cells that inhabits the skin and contributes to skin function and renewal. Adult stem cells are best defined by their capacity to self-renew, and to maintain tissue function for a long period. Keratinocyte stem cells, like hematopoietic stem cells, match this functional and rigorous definition. Moreover, unipotent (single-lineage) and multipotent (multiple-lineages) keratinocyte stem cells coexist in adult skin. Compiling evidence demonstrates that the basal cell layer of the epidermis contains unipotent keratinocyte stem cells that are each responsible for the renewal of a tiny portion of the epidermis. Multipotent keratinocyte stem cells are located in the upper region of the hair follicle below the sebaceous glands, but sweat glands and sweat ducts may also contain multipotent keratinocytes (our unpublished results). Unipotent and multipotent stem cells are clearly related to each other. Indeed, keratinocytes can migrate out of a hair follicle or a sweat gland, to adopt an epidermal fate and to regenerate a wounded epidermis. Most importantly, keratinocyte stem cells, which can divide every 16 to 18 hours, have a remarkable growth capacity in culture, far better than that of dermal fibroblasts. This remarkable growth capacity is at the origin of cutaneous cell therapy.

Keratinocyte stem cells have other remarkable, although circumstantial, properties. They divide infrequently *in vivo*, in contrast to their transient amplifying progeny, which multiply rapidly, and they express high levels of β1 and α6 integrin, and low levels of CD71 (transferrin receptor). Theoretically, a keratinocyte stem cell can generate a progeny with variable fate depending on circumstances, the most probable fates being those of a stem cell (symmetrical divisions), of a transient amplifying cell, or both (asymmetrical division). However, more research is needed to fully-comprehend the mechanisms involved in stem cell decision-making.

Clonal Analysis

The growth potential of individual multiplying keratinocytes is best evaluated by clonal analysis, which can be performed either with cells freshly isolated from the skin or with cultured cells. A clonal analysis experiment typically implies the isolation and the cultivation of 100 to 200 individual cells. Briefly, each single cell is isolated with an elongated Pasteur pipette, under direct vision using an inverted microscope, before it is carefully deposited at the centre of a 35 mm Petri dish containing lethally irradiated 3T3 cells. After seven days of cultivation, each dish is carefully searched for the presence of a clone under an inverted microscope. Each clone is then dissociated with trypsin to obtain a single cell suspension, which is inoculated into several indicator dishes (usually 2 × 100 mm Petri dishes) containing lethally irradiated 3T3 cells. Cultures are fixed after 12 days of cultivation, stained with Rhodamine B, and examined under a dissecting microscope (Figure 61-2). Three types of colonies are easily distinguished: (1) growing

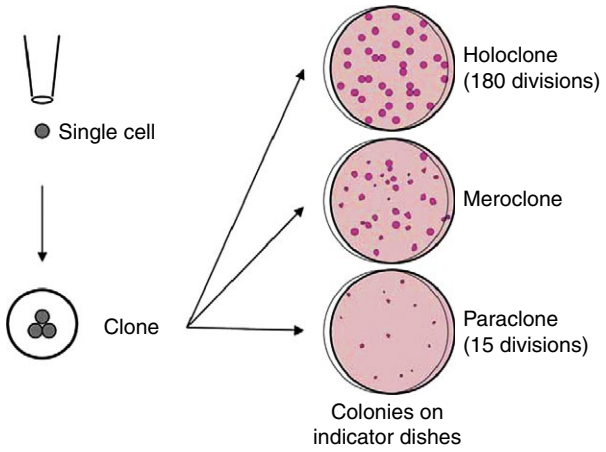


FIGURE 61-2 Clonal types. A single cell is isolated with a Pasteur pipette and cultivated onto a feeder layer of irradiated 3T3 cells. If a clone forms, it is passaged while still growing exponentially. The number and shape of colonies on the indicator dishes determine the clonal type of the founding cell. The progeny of a holoclone generates large, progressively-growing colonies, the progeny of a meroclone generates a mixture of large, progressively-growing colonies, and aborted colonies, and that of a paraclone generates only aborted colonies or no colony. The proportion of those clonal types varies with donor age. Fetal skin mostly contains holoclones, young skin mostly contains holoclones and meroclones, and old skin mainly contains meroclones and paraclones.

colonies that are large and round, have a smooth perimeter, and mostly contain small cells. These colonies can be passaged many times, and are formed by keratinocytes with significant growth potential. (2) Aborted colonies are small, have an irregular shape, and contain large, flattened differentiated squame-like cells. These colonies cannot be passaged, and are formed by cells with restricted growth potential. (3) Mixed colonies usually have a jagged perimeter, and contain intermingled areas of small and large differentiated cells. These colonies can only be passaged a limited number of times, and are formed by cells with limited growth potential.

Clonal Types

The growth potential of a clonogenic keratinocyte is best evaluated by the frequency of aborted colonies formed by its progeny in indicator dishes (Figure 61-2). A cell whose progeny forms almost exclusively growing colonies is termed "holoclone." A cell whose progeny forms growing, mixed colonies, and aborted colonies, is termed "meroclone," and a cell whose progeny forms exclusively aborted colonies or no colony is termed "paraclone." Serial cultivation experiments have demonstrated the tremendous growth capacity of holoclones (up to 180 divisions), whereas transplantation experiments have demonstrated the ability of holoclones to form an epidermis. Indeed, a single holoclone can generate a large enough progeny to entirely restore the epidermis of an adult human. Moreover, clonal analysis experiments have demonstrated the self-renewal capacity of holoclones, in culture and *in vivo* (our unpublished results). Hence, holoclones fulfill the fundamental definition of adult stem cells. The epithelial cells of stratified squamous epithelia are all termed keratinocytes, even if the latter differ in their differentiation capacities. Therefore, all stratified epithelia contain holoclones that theoretically should be referred to as epidermal holoclones, vaginal holoclones, or limbal holoclones. Most importantly, the term holoclone only refers to the growth capacity of a keratinocyte, and not to the lineage capability of the cell. Therefore, some holoclones are multipotent (i.e., follicular holoclones), whereas others are unipotent (i.e., epidermal holoclones). Meroclones, whose growth potential is always lower than that of holoclones, are best characterized by an increased capacity to generate paraclones, possibly by asymmetrical division. Most importantly, the growth potential of meroclones directly correlates with the frequency of paraclone formation. Meroclones with a significant growth potential generate few paraclones, and can form an epidermis when transplanted. Meroclones that generate a significant number of paraclones have a limited growth potential, and are unlikely to contribute to long-term renewal of the epidermis. Thus, meroclones are not strictly self-renewing, and can be viewed as the skin equivalent of short-term hematopoietic stem cells. The growth potential of paraclones is restricted to 1–15 divisions, meaning that the size of the progeny of a paraclone ranges between two cells and 32 000 cells. Consequently, paraclones are transient amplifying cells. However, paraclones are unlikely to be the transient amplifying cells identified *in vivo* by kinetic experiments. Indeed, the number of paraclones in a suspension of freshly-isolated keratinocytes, whatever the age of the donor, is far below the expected number of transient amplifying cells (our unpublished results), which supposedly includes most multiplying cells in the epidermis. Paraclones may then be a population of multiplying cells, generated in response to special circumstances such as wound healing and cultivation.

Clonal Conversion

A culture of human keratinocytes is thus composed of cells with differing capacity for growth, which are termed paraclone, meroclone, and holoclone, are best identified by clonal analysis. Clonal analysis experiments demonstrate that holoclones self-renew for a few passages in culture, but they stop self-renewing for unknown reasons at some point and convert entirely to meroclones, which in turn generate paraclones (our unpublished results). Ultimately, the culture ends without multiplying cells. This phenomenon is termed "clonal conversion" (Figure 61-3). The conversion of holoclone to meroclone, and to paraclone, is irreversible under normal conditions, and is greatly influenced by the conditions of cultivation. For instance,

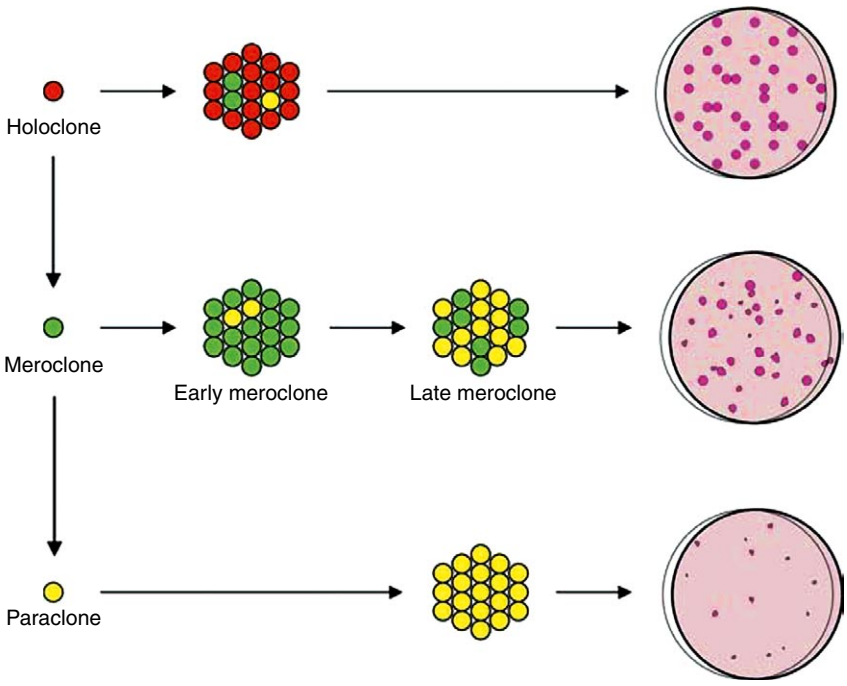


FIGURE 61-3 Clonal conversion. A holoclone generates holoclones, few meroclones, and almost no paraclones. A meroclone generates more paraclones with time. A paraclone generates only aborted colonies. Clonal conversion increases with passages, and is drastically enhanced by suboptimal culture conditions.

it takes more than 23 weeks to exhaust the growth potential of a holoclone cultivated onto a feeder layer of irradiated 3T3 cells in presence of fetal calf serum, an otherwise impossible achievement with suboptimal culture conditions or in defined medium. It is why culture conditions of keratinocytes must be constantly monitored to maintain the highest level of cultivation, especially when the cells are to be transplanted. Clonal conversion is also accelerated by many parameters, including age. In our hands, the number of paraclones in a suspension of freshly-isolated keratinocytes is independent of donor age, but becomes age-related after the primary keratinocytes are passaged (our unpublished data). Indeed, secondary cultures initiated from aged donors contain many more paraclones than cultures initiated from young donors, and they cannot be passaged as many times. Hence, the formation of paraclones reveals the effect on cell growth of age-related genetic modification, or that of an adverse epigenetic phenomenon. This is of paramount importance when cells are cultivated for therapy, as clonal conversion can adversely affect the engraftment (take) of cultured epithelium grafts, and the long-term renewal of the regenerated epidermis.

Keratinocyte Cultivation

Generalities

In 1975, Rheinwald and Green reported the cultivation of human keratinocytes using a feeder layer of irradiated 3T3 cells in a seminal paper, and envisioned its potential application for the treatment of extensive skin defects. It took Green and his colleagues eight more years of intensive work before the first transplant of cultured autologous keratinocytes onto a human was accomplished. This period was paved with several landmarks, including the use of the enzyme dispase to detach the cultured epithelium as a transplantable homogenous sheet, and the demonstration that cultured human keratinocytes can generate an epidermis once transplanted onto athymic mice. The technology has been successfully used worldwide to treat hundreds of extensively burned patients. Green's method to cultivate transplantable keratinocytes is extremely efficient, and we have always been able to initiate cultures, even from tiny biopsies. The cultivation of epidermal stem cells for therapy requires the use of a feeder layer of lethally irradiated mouse 3T3 cells and serum. To our knowledge, no clinical success has been obtained with cells grown in defined medium or in other conditions. The use of cells of animal origin (xenotransplantation) can theoretically induce adverse reactions and transmit pathogens. However, the risk is significantly minimized by using 3T3 cells originating from a master bank that tested negative for a battery of viruses, according to guidelines of regulatory agencies. Animal serum, widely used in the biotechnology industry, is also a potential life hazard, both through the induction of adverse reactions to foreign proteins, and the transmission of pathogens. Again, the risk is minimal if lots of serum with traceable origin, and for which the manufacturer maintains a master file in accordance with the guidelines of regulatory agencies, are purchased.

Most importantly, no adverse reaction to 3T3 cells and serum has been reported in the hundreds of patients treated with epidermal stem cells cultivated in the presence of serum and 3T3 cells. The monitoring of culture conditions include the testing of serum lots, media, and supplements,

and is best accomplished by using a reference strain of keratinocytes, the growth characteristics of which are well-known. Indeed, any modification in the method of cultivation (e.g., a new lot of serum or supplements) should be systematically tested in a colony-forming assay using a reference strain, to determine the extent to which the modification affects the ratio of growing colonies (stem cells and close progeny) to aborted colonies (paraclones). It also necessitates strict adherence to instructions and protocols. For instance, 3T3 cells are best grown in the presence of bovine serum, and switching to fetal bovine serum is a common mistake. In our experience, fiddling with protocols in an uncontrolled manner rarely results in better culture performances, and often has deleterious consequences, such as enhanced clonal conversion resulting in rapid exhaustion of growth capacity of the stem cells and, consequently, in failure of engraftment.

A cutaneous wound ruptures skin integrity, and can have serious consequences, such as infection, loss of proteins, and even death. Moreover, chronic wounds favor improper scarring and the development of skin cancers. Burn wounds are a perfect illustration (Figure 61-1). A first-degree burn wound which only affects the epidermis heals spontaneously, and without a scar. Reconstitution of the epidermal integrity then results from the migration of keratinocytes originating from the nearby epidermis or appendages. A second-degree burn wound, defined as superficial or deep on the depth of the dermal burn, destroys the epidermis and part of the dermis. It heals spontaneously by the migration of cells originating from the epidermal appendages. However, a permanent scar will form. A third-degree burn destroys the entire thickness of the skin (full-thickness burn). Although a tiny third-degree burn wound can possibly heal with a scar by migration of cells from the surrounding unwounded skin, a large one will not. The transplantation of split-thickness autografts obtained from unburned donor areas is then the unique therapeutic option, with the added risk of improper healing of donor sites, including infection, hypertrophic scars, and cheloids. Moreover, the recurrent cropping of donor sites can become a serious problem, especially for patients burned over 60% of the total body surface area (TBSA). Progress in medical resuscitation and intensive care allows patients whose skin has almost completely burned, to remain alive. The transplantation of autologous epidermal stem cells then becomes life-saving for those patients who do not have enough donor skin for conventional split thickness autografts.

Methodology

The methodology has been reported in great detail. Briefly, a full thickness skin biopsy is obtained aseptically from normal skin, to isolate stem cells from both the epidermis and the epidermal appendages. The biopsy is then stored in culture medium and transferred to the laboratory, where it is minced with scissors and treated with trypsin to obtain a single-cell suspension. The cells are seeded at a density of 2.4×10^4 cells/cm^2 onto a feeder layer of irradiated 3T3 cells in medium containing fetal bovine serum, and supplemented with cholera toxin, hydrocortisone, insulin, and triodothyronine, according to published protocols. Cultures are supplemented with epidermal growth factor (10 ng/ml) at the first medium change. Under these conditions, clonogenic keratinocytes divide every 16 to 18 hours, and colonies quickly expand and push away the irradiated 3T3 cells, which then detach from the culture vessel. If the culture lasts long enough, the colonies of keratinocytes will fuse and form a stratified epithelium. The method of cultivation selects for clonogenic keratinocytes, but some melanocytes and dermal fibroblasts can also adhere and multiply, although at a much slower rate than the keratinocytes. Primary cultures are usually passaged while still in exponential growth—i.e., before confluence, or after nine days of cultivation, whichever comes first. If primary cultures yield enough cells, cultures for transplantation are then initiated at a seeding density of 1.2×10^4 cells/cm^2, and the leftover cells are frozen for future use. If the primary cultures do not yield enough cells, it is then necessary to further expand the cells by setting up secondary cultures at a seeding density of 1.2×10^3 cells/cm^2. Cells are then cultivated for no more than a week before they are used to set-up cultures for transplantation. Under optimal conditions, a 10 000-fold expansion is obtained within two to three weeks. Confluent cultured epithelia then need to be detached enzymatically from the bottom of the culture vessels, using dispase or thermolysine. However, the enzymatic treatment significantly shrinks the cultured epithelium. Once rinsed, each epithelial graft is mounted onto petroleum gauze backing, with special care being taken to maintain the correct orientation of the epithelium, (i.e., the basal layer facing away from the backing). This step of the procedure is cumbersome, tedious, and manpower-demanding, especially when hundreds of cultured epidermal autografts (CEAs) need to be prepared in a reasonable amount of time. This is why many groups have explored alternative methods to cultivating keratinocytes.

Transplantable Matrices

Clonogenic human keratinocytes can be cultivated with variable success on various matrices, including human fibrin, bovine or human collagen; hyaluronic acid; shark proteoglycans; and polymers. Therefore, it is crucial to thoroughly evaluate the effects of each of these matrices on the growth of the keratinocyte stem cells, since suboptimal culture conditions may result in enhanced clonal conversion, a reduced keratinocyte lifespan, and an irreversible degradation of the quality of the graft. This is best accomplished by clonal analysis and by transplantation of the cultured cells onto athymic or severe-combined immunodeficiency mice, using reference strains of keratinocytes, the performances of which are well-known. Using this approach, we have

demonstrated that fibrin matrices alleviate the need for the detachment of the grafts, eliminate shrinkage, and hence have considerable advantages over plastic for the culture of epidermal stem cells for grafting. Use of a fibrin matrix considerably shortens the duration of cultivation required to generate many CEAs, making it possible to transplant the entire body area of an adult human with CEAs as soon as 15 days after injury. Most importantly, the long-term regeneration of human epidermis on third degree burns transplanted with autologous cultured epithelium grown on a fibrin matrix has been demonstrated. Fibrin matrices are also extremely useful for transplantation of autologous limbal stem cells to restore a corneal epithelium.

The transplantation of small colonies of keratinocytes (preconfluent cultures) grown on fibrin matrices, or of individual keratinocytes suspended in fibrin gels has been proposed. This may be useful to quickly transplant small full-thickness burns, or to enhance the healing of superficial burn wounds. However, it remains to be demonstrated that individual cells or small colonies generate an epidermal barrier faster than confluent and organized cultures when directly exposed to the hostile environment of the grafting bed in large full-thickness burns. Composite grafts combining autologous keratinocytes and fibroblasts embedded in a collagen gel, a fibrin matrix, or polymers may also be useful to generate a closer-to-normal skin by enhancing the formation of dermis. However, with today's technology, the population of fibroblasts in a small skin biopsy cannot be sufficiently expanded in a timeframe compatible with the transplantation of acute burn wounds. This is why composite grafts, associating autologous fibroblasts and keratinocytes, are reserved for chronic wounds. Many groups have proposed using allogeneic fibroblasts, large stocks of which can be prepared in advance. However, allogeneic fibroblasts do not permanently engraft, despite contradictory claims in the literature. To our knowledge, composite grafts may be efficient to treat chronic skin defects such as leg ulcers, but they have not proven useful for the successful treatment of extensive burn wounds. Hopefully, progress in the cultivation of human fibroblasts will be made; then, composite grafting will have a major impact.

Transplantation of Keratinocyte Stem Cells

Various surgical procedures have been developed over the years to transplant cultured keratinocytes. CEA were initially transplanted directly on the granulation tissue that spontaneously forms after full-thickness burns were excised to fascia. Success was then variable, ranging from excellent to poor, and surgeons quickly stressed that the quality of the wound bed was crucial. In that regard, one of the most appreciated procedures is known as Cuono's, in which full-thickness burns are excised to fascia, and temporarily covered with meshed, expanded, fresh or cryopreserved split-thickness human allografts obtained from organ donors. At grafting time, which usually occurs two to three weeks after the initial biopsy was taken for expansion of stem cells, allografts are dermabraded or tangentially excised, and a careful hemostasis is performed. Cultured epithelium grafts, with the basal cells oriented toward the grafting bed, are then gently applied and covered with petroleum gauze dressings to prevent desiccation (Figure 61-4). Sliding and displacement of the graft should be strictly avoided. CEA are then overlaid with bridal veil gauze, sterile dry compresses, and elastic bandage. Skin substitutes like Integra® or Biobrane® are useful alternatives to cadaver skin, when the latter is not available or cannot be used for religious reasons.

Cuono's procedure has greatly increased the rate of CEA engraftment, which is best-appreciated clinically. At the time of the first dressing ("take-down"), the engrafted epithelium appears shiny and translucid. With time, the regenerated epithelium matures to resemble a normal epidermis (Figures 61-4 and 61-5). CEAs perform best in areas in which shear forces or frictions are minimal, and in non-weight-bearing surfaces. Successful engraftment now averages 70% of the transplanted area, but it is not unusual to obtain a 100% take. In a recent study, younger age was significantly associated with better CEA take, possibly reflecting clonal type differences between young and old people. Most importantly, the transplantation of CEA necessitates highly-trained surgical and nursing staff. For instance, four to six nurses require from 60 to 90 minutes to perform each dressing change following CEA application at the Percy Burn Center in France, which has one of the largest and most successful records in CEA transplantation. Infection of the grafting bed is the enemy of CEAs, and provokes a quick disappearance of the grafts. The fight of infection relies on systemic antibiotics and local antiseptics, which are clinically efficient, and usually not harmful to the CEAs, despite an alleged toxicity *in vitro*. Fragility and blistering of CEAs are also commonly observed during the weeks following transplantation, most likely as a consequence of a delayed maturation of the anchoring fibrils. In some cases, lysis of CEAs, which had otherwise nicely engrafted, occurs after a few weeks with no rational explanation. Bleeding of the grafting bed can also result in loss of CEA, further emphasizing the importance of thorough hemostasis at grafting time. Another complication of CEAs is epidermal hyperkeratosis, which may need repeated treatment with moisturizing and keratolytic ointments. Wound contracture is also a problem, as in all patients with large burns, but there seems to be less hypertrophic scarring with CEAs than with conventional split-thickness autografts.

Regeneration of Epidermis

Cultured epithelia prepared according to the methods of Green and colleagues are stratified, but lack the uppermost-differentiated epidermal layers necessary for barrier function.

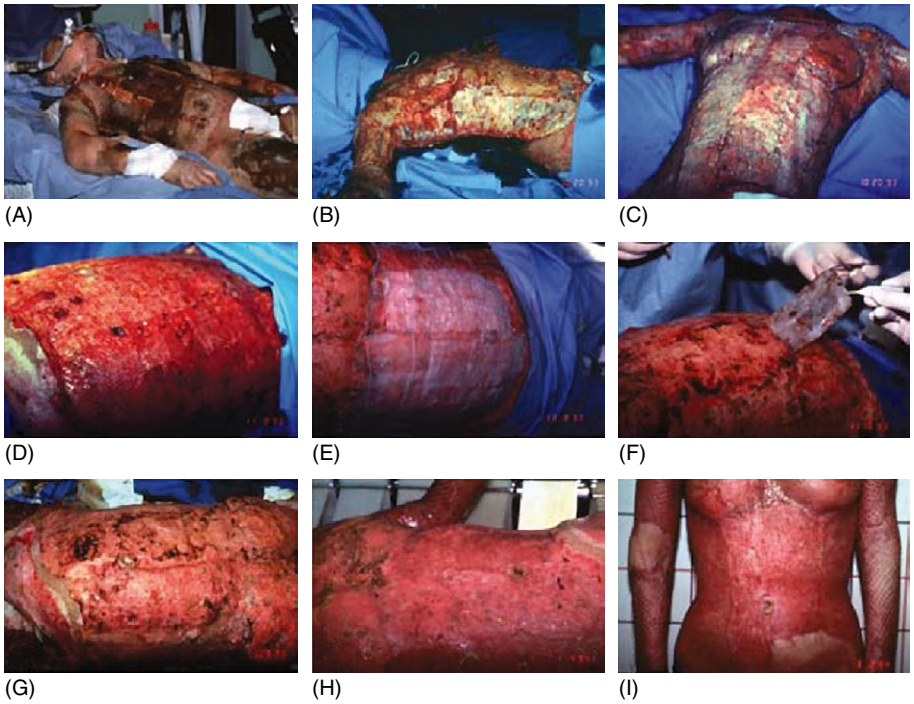


FIGURE 61-4 Transplantation of human cultured epithelium autografts. A 35-year-old woman was burned by flames; over 50% of her body has third degree burns. (A) Admission; (B) Excision to fascia of full-thickness thoracic and abdominal burn wounds the day after injury. (C) Temporary coverage of the excised area with split-thickness meshed human cadaver allografts. (D) Appearance of the dermabraded grafting bed. (E) Transplantation of control cultured epithelia (top) and cultured epithelia grown on fibrin matrices (bottom). (F) Appearance of the transplanted area eight days later (takedown). (G) Appearance of the same area 21 days later. Note the excellent take of both control (bottom) and experimental (top) cultured epithelia (the photograph was taken from the opposite side to that shown in panel (E)). (H, I) Appearance of the same transplanted area 57 days and four months later. Reproduced with permission from Ronfard *et al.*, (2000).

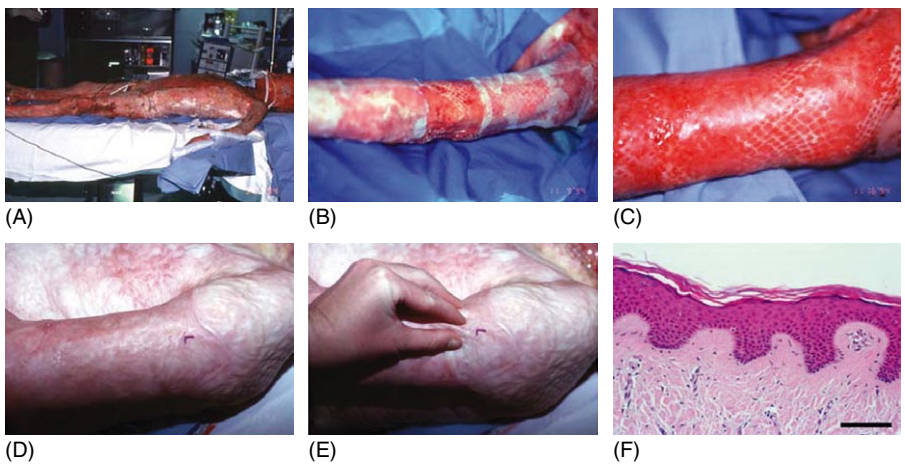


FIGURE 61-5 Long term follow-up of cultured epithelia transplanted on a fibrin matrix. A nine-year-old boy was burned by flames; over 95% of his body has third degree burns. (A) Admission at Percy Burn Centre a month after injury. (B) Transplantation of cultured epithelia grown on a fibrin matrix on the left arm. (C) Appearance of the transplanted area at takedown. (D, E) Clinical appearance of the skin 3.5 years after the transplantation. The skin is elastic when pinched, and has a smoother appearance than the neighbouring split-thickness skin autografts. (F) Histological appearance of the skin 3.5 years after transplantation. The epidermis is histologically normal. Note the presence of rete ridges, and a superficial neodermis with vascular arcades. Similar results were obtained with cultured epithelia grown in absence of fibrin. Bar: 100 μm. Reproduced with permission from Ronfard *et al.*, (2000).

However, the horny layers mature rapidly once the CEA are transplanted, indicating that the cells adequately express their program of differentiation. This epidermis always contains suprabasal Langerhans cells, and may also contain melanocytes, although at a variable density in the basal layer. The presence of Merkel cells has been reported. The most surprising observation is the presence of rete ridges, vascular arcades, and elastic fibers, which are normally not

observed during normal wound healing. However, the formation of a neodermis that closely resembles the papillary dermis is a slow process that takes years (see Figure 61-5). This extremely intriguing observation suggests that mesenchymal stem cells may have been transplanted along with the epidermal stem cells, and that epidermo-mesenchymal interactions reminiscent of fetal wound healing have occurred. This awaits confirmation. Most importantly, hair follicles and sweat glands are not regenerated. There are several explanations for this: multipotent epithelial stem cells are not present in CEAs, possibly because current culture conditions favor the epidermal lineage, or because multipotent stem cells are absent in adult skin, and the inductive signals necessary for appendage morphogenesis are missing. However, recent results from our laboratory indicate that the skin of adult mammals contains multipotent epithelial cells with the capacity to generate epidermis, sebaceous glands, and all follicular lineages. Other groups have demonstrated that dermal papilla cells obtained from adult hair follicles have inductive capacities. These are important findings that open the path to the reconstruction of hair follicles and other epidermal appendages.

The long-term self-renewal of epidermis generated from CEA is a concern, in view of recent findings demonstrating a shortening of telomeres in keratinocytes obtained from transplanted patients. This further illustrates that cells should be cultivated according to state-of-the-art technology, and that cell culture, as good as it can be, is a stress to cells. It is impossible as yet to distinguish between the consequences of clonal conversion, which can occur very quickly in relation to poor cultivation and independently of the mitotic clock, and the shortening of telomeres, a difference expressed in a recent review as mitotic clock or culture shock. The expansion of the epidermal stem cells in culture represents at the most 22 cell doublings, a small number when holoclones can undergo at least 180 doublings, meaning that holoclones and early meroclones have plenty of growth potential left to renew their share of epidermis for a long time. The number of stem cells (holoclones) or cells with significant growth potential (meroclones) present in the CEAs, that can engraft and self-renew the epidermis becomes important. Surprisingly, there are no data in the literature on this subject, even though hundreds of patients have been transplanted. Nevertheless, clinical follow-up indicates that epidermis generated from CEAs self-renews for years, suggesting that holoclones and meroclones are present in sufficient numbers.

Indications of Cultured Epidermal Autografts

Cell Therapy

Clinical indications of CEAs have been extensively reviewed. The main indications for CEA are full thickness burns over 60% of TBSA, in which CEAs are highly beneficial and lifesaving. CEAs can also be useful in reconstructive surgery to enhance the healing of donor sites, to prevent recurrence of cheloids, and to cover wounds made by the excision of giant congenital nevi, and of tattoos. CEAs may also be useful in urology and oral surgery. But one of the most impressive uses of CEAs is in ophthalmology. Pellegrini and colleagues have beautifully demonstrated that limbal stem cells (limbal holoclones) can be cultivated on a fibrin matrix and transplanted to restore the integrity of the corneal epithelium. This approach, coupled with elective keratoplasty, has restored vision to patients whose sight was impaired because of corneal burn wounds.

Niche market, customized manufacturing, and high costs have made it difficult for CEAs to become a blockbuster product. As an example, Epicel®, a commercial CEA manufactured according to good manufacturing practices is considered a humanitarian device by the Food and Drug Administration (www.genzyme.com); it costs about $14 US per cm^2 (including biopsy kit, culture, and shipping), which prices the transplantation of a square meter of CEA at $140 000 US. However, this cost should be compared to that of other transplant procedures. Nonetheless, progress has been made. For instance, use of a fibrin matrix that diminishes the fragility of the CEA, and is user-friendly, should reduce costs as the number of CEAs necessary to cover an area is two-fold lower, the duration of cultivation is significantly shortened, and there are no large personnel requirements to prepare the grafts. Composite grafts, including autologous mesenchymal and keratinocyte stem cells, are also foreseen, but they are more complex to manufacture, and their cost will certainly be higher than that of currently available CEAs.

Ex Vivo Gene Therapy

Gene therapy of hereditary disabling diseases of the skin or of the cornea will certainly benefit from the experience gained with the treatment of large burns. Indeed, epidermal stem cells or limbal stem cells can be successfully transduced by defective retroviral or lentiviral vectors, and engineered to express a protein of medical interest. For instance, transduction of a cDNA-encoding laminin 5 or collagen VII in epidermal stem cells of patients with junctional epidermolysis bullosa or dystrophic epidermolysis bullosa, respectively, should permit the treatment of elective areas to enhance the quality of life of those patients. Mavilio and De Luca's groups have recently demonstrated the feasibility of *ex vivo* gene therapy. In this breakthrough, the authors used a straightforward retroviral strategy to stably transduce a *LAMB3* cDNA encoding laminin 5 β3-chain into keratinocyte stem cells of a patient with a rare form of junctional epidermolysis bullosa (JEB), a devastating skin disease characterized by skin fragility, chronic blistering, painful wounds, and the emergence of

carcinomas. Genetically corrected *LAMB3* CEA were then produced and transplanted to restore epidermal integrity. Importantly, the transplanted areas have remained healed since (De Luca, personal communication), unambiguously demonstrating stem cell engraftment. Mastering the cultivation and the transplantation of keratinocyte stem cells was assuredly the key to success, as the authors have thorough experience in transplanting cultivated keratinocyte stem cells to treat burns or corneal defects. The choice to transplant chronic non-healing wounds depleted in resident stem cells was also crucial, since it likely removed competition between those stem cells present in skin appendages (i.e., in hair follicles and sweat glands) and the transplanted stem cells.

Despite the fact that the transplantation of autologous cultivated keratinocytes has contributed to save the lives of thousands of patients worldwide, some have questioned the origin of the healing cells, suggesting that stem cells other than epidermal (e.g., hematopoietic stem cells) were responsible for epidermal reconstitution in large burns. Mavilio's data, together with our own data demonstrating that the progeny of a single EGFP keratinocyte multipotent stem cell of the rat can reconstitute hair follicles and sebaceous glands for years in serial transplantation, should definitely close the argument on whether keratinocyte stemness is maintained in culture. Another important finding of Mavilio's is that few stem cells are necessary to reconstruct and renew a recombinant epidermis. This opens the door to the transplantation of a genetically-homogenous CEA produced from a single transduced holoclone, in which one can demonstrate safe integration of the recombinant provirus; this together with the use of a self-inactivating shuttle vector should further contribute to safe epidermal gene therapy.

FUTURE DEVELOPMENT

A promising development is the derivation of keratinocytes from embryonic stem cells (ESCs) and, interestingly, the transcription factor p63 appears important for epidermal commitment. Several groups have demonstrated the feasibility of the approach in the mouse and the human, but the ESC-derived keratinocytes, at least in the human, do not yet seem to fully recapitulate the properties expected from adult keratinocyte stem cells. Another exciting development is the recent derivation of induced pluripotent stem cells (iPS) from adult human keratinocytes, which, in combination with the isolation of keratinocytes from a small skin biopsy or even a plug hair, is a powerful means to obtain customized iPS cell lines. Nevertheless, the derivation of keratinocytes from ES cells or iPS does not preclude mastering the cultivation and transplantation of adult keratinocyte stem cells, and it surely does not alleviate the problem of engraftment.

SUMMARY

A challenge in *ex vivo* skin cell therapy is to obtain a massive expansion of the stem cells from a small skin biopsy in the shortest time possible, while preserving stemness. The method of cultivation described by Rheinwald and Green in the seventies achieves this goal, and it has not been matched. Cell therapy using autologous keratinocyte stem cells, cultivated according to the preceding method, has saved the lives of hundreds of burned patients, has restored sight to vision-impaired patients, and has opened the door to *ex vivo* gene therapy. However, the technology relies on a feeder layer of irradiated mouse cells, and is therefore considered a xenotransplantation by regulatory agencies, which adds a significant burden to an already cumbersome approval process. Hence, one has to improve the cultivation of keratinocyte stem cells (and also that of skin mesenchymal stem cells), to improve engraftment, to reconstruct hair follicles and sweat glands, and to improve the mechanical properties and the esthetic of the regenerated skin. The answer to these challenges resides in a better understanding of the cellular and molecular events involved in skin renewal and morphogenesis; in a better understanding of fetal and adult wound healing; in that respect, embryonic stem cell lines might be helpful. It is also of paramount importance to study stem cell engraftment in animal models, e.g., the pig, the skin biology of which is closer to the human than that of rodent. In conclusion, future progress in skin cell therapy necessitates: (1) continuous scientific knowledge to improve stem cell engraftment and transplant functionality; (2) the development of a cultivation technology that meets regulatory requirements without affecting stemness; and (3) a thorough evaluation of the economic constraint, as it can affect the commercial development of cell therapy.

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KEY WORDS

Clonal conversion Conversion of a holoclone to a meroclone or to a paraclone irreversible under normal conditions; affected by culture conditions and age.

Holoclone (Gk *holos* whole + Gk *klôn* growing) Colony-forming cell with tremendous growth potential (up to 180 divisions), whose progeny forms almost exclusively growing colonies.

Meroclone (Gk *mero* part + Gk *klôn* growing) Colony-forming cell whose growth potential is lower than the

growth potential of a holoclone but superior to 15 divisions; forms growing and aborted colonies.

Paraclone (Gk para *akin* + Gk *klôn* growing) Colony-forming cell committed to a maximum of 15 divisions; forms only aborted colonies.

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Chapter 62

Orthopedic Applications of Stem Cells

Jerry I. Huang and Victor M. Goldberg

INTRODUCTION

More than 33 million musculoskeletal injuries occur in the United States each year. Bone is capable of regeneration, and defects often heal spontaneously. However, cartilage, tendon, and ligament injuries usually result in replacement of the site by organized scar tissue, which is inferior to the native tissue. An increased understanding of cell biology and various tissue types may lead to the future possibility of using tissue engineering techniques to recapitulate the embryonic events that result in the development of native tissue. The goal of tissue engineering is to generate biological substitutes for repair or replacement of injured tissue. Three basic elements are required. First, appropriate cells must be present to give rise to the structural tissue. Second, appropriate growth factors and differentiation stimuli must exist for the cells to proceed down the proper lineage. Third, a scaffolding matrix must act as a building block for cellular attachment, differentiation, and maturation into the desired tissue. The generated construct must be site-specific, and integrate well at the host–graft interface.

Technological advances have resulted in the advent of a wide array of biomaterials and synthetic growth factors. Clinicians and scientists face the enormous task of generating biological constructs that emulate the interaction between cells and the complex extracellular matrix they secrete that encapsulates them. A thorough understanding of the biology of each tissue type (material properties, ratio of extracellular matrix components, and cellular profile) is essential to the construction of functional tissue. The first element of tissue engineering is the use of cells. Different approaches include the use of differentiated lineage-specific cells (osteoblasts, chondrocytes, tenocytes, meniscal fibrochondrocytes, etc.), or the use of progenitor cells.

Pluripotent mesenchymal stem cells (MSCs) with the ability to differentiate into multiple mesodermal lineages have been isolated from bone marrow, adipose tissue, skeletal muscle, and synovium. Recently, they have also been isolated from umbilical cord blood and placental tissue. Stem cells have the advantage that they are unlimited in supply, easily harvested, and can be expanded in tissue culture to large numbers. Moreover, it is postulated that induction of stem cells down various mesodermal lineages will result in tissue that more closely resemble native tissue, and recapitulate embryonic development. MSCs isolated from human bone marrow aspirates have been shown to retain pluripotentiality and proliferative ability through long-term passaging. Moreover, although the proportion of regenerating MSC titers decreases with advancing age, the capacity of MSCs to differentiate into bone, cartilage, and other mesodermal tissue types is independent of the age of the donors.

Another advantage of using MSCs for tissue engineering strategies is that they can be used in allogeneic transplantation. Human MSCs cells do not induce a mixed lymphocyte reaction when incubated with allogeneic donor lymphocytes, and have the ability to suppress an ongoing mixed lymphocyte reaction. In fact, MSCs have been shown to be effective as immunomodulators in inhibiting graft-versus-host disease. Allogeneic MSC-based tissue constructs would be limitless in supply, and have enormous economic advantages.

A number of growth factors have been described that enhance angiogenesis, promote cell proliferation, and induce differentiation of cells down various mesodermal lineages. The delivery of single doses of recombinant bone morphogenetic proteins has been demonstrated to be effective in the healing of segmental bone defects and articular cartilage defects. Similarly, the structural properties of ligaments during the repair process can be improved by the application of growth factors. However, in many clinical situations a sustained exposure to growth factors is necessary. Moreover, leakage of supraphysiological doses to adjacent sites can pose huge problems, such as heterotopic ossification from osteoinductive factors.

In addition to being a powerful tool in cell-based therapy, MSCs may also be useful as a delivery vehicle for growth factors through genetic manipulation. Cells can

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be transduced with a retrovirus, so that the target gene can be integrated into the cell's DNA. This allows for propagation of gene expression when the cell replicates, so that long-term expression of the target gene would occur. Alternatively, gene therapy strategies using adenoviral vectors would be useful in clinical situations where only transient gene expression is desired. *In vivo* gene therapy, in which the viral vector is introduced to a tissue by direct injection, has many disadvantages. A host response to the virus may result in immunological rejection. Moreover, transduction efficiencies are often low, especially if the target tissue contains slowly-growing cells. Diffusion of the vector to adjacent areas may also lead to potential complications. *Ex vivo* gene therapy using MSCs overcomes some of these potential problems. Use of autologous cells as delivery vehicles would prevent the host immune response. In addition, cells can be seeded onto matrix scaffolds customized to the shapes and sizes required at the target tissue.

This chapter provides a brief summary of the biology and properties of various musculoskeletal tissue types, highlights the present tissue engineering strategies, and draws speculations as to the future direction of the application of MSCs in tissue engineering as it applies to each tissue. MSCs can serve as the building blocks for tissue regeneration by their ability to differentiate down various lineages. They also hold great promise as delivery vehicles for sustained release of growth factors to injured tissue sites. The growth factors may enhance tissue repair by promoting migration of MSCs, inducing differentiation of progenitor cells, and enhancing vascularization of the newly-formed tissue.

BONE

Bone has the ability to regenerate itself with functional tissue possessing properties similar to the original tissue. However, bone tissue engineering still has many clinical applications, including fracture nonunion, congenital malformations requiring bone-lengthening, tumors, and bone loss secondary to trauma or osseous infections. Moreover, bone regeneration in the setting of bony ingrowth is important in joint arthrodesis for osteoarthritis, spinal fusion, and more rigid fixation of prosthetic implants.

The extracellular matrix of bone is primarily type I collagen and calcium phosphate. Osteoblasts line the periphery, and are active in matrix deposition. In the clinical setting, autogenous bone grafts harvested from the iliac crest and fibular grafts are often used in fracture repair. However, some of the disadvantages of harvesting autogenous bone graft are donor-site morbidity, and their limited supply. Clearly, there is a need for alternative methods of harvesting autologous bone substitutes. The efficacy of percutaneous bone marrow grafting was demonstrated in a series of 20 patients with established tibial nonunions. Under local anesthesia, bone marrow was aspirated from the iliac crest and injected directly into the nonunion site. Clinical and radiographical bony union occurred in 75% of patients at a mean of 14 weeks. Percutaneous harvest of bone marrow aspirates is minimally invasive, and can be performed under local anesthesia. Bone marrow suspensions may serve as useful substitutes for traditional bone graft harvests. The effectiveness of this technique is dependent on the number of progenitor cells in the cellular suspension. With the use of a cell separator, which concentrates the progenitor cells into a more compact volume, bony healing was obtained by Hernigou *et al.* in 53 of 60 patients with noninfected atrophic tibial nonunions.

MSCs isolated from bone marrow aspirates and processed lipoaspirate (PLA) cells from adipose tissue differentiate into osteoblasts when cultured in the presence of dexamethasone, ascorbic acid, and beta-glycerophosphate. Bone marrow-derived MSCs have also shown the ability to form heterotopic bone in animal models. MSCs seeded onto hydroxyapatite (HA) and tricalcium phosphate (TCP) ceramic discs implanted into subcutaneous pockets show evidence of bone formation within the pores of the ceramic scaffolds. Yoshikawa *et al.* demonstrated that preincubation of human MSCs in porous ceramics in the presence of osteoinductive medium, followed by intraperitoneal implantation into athymic nude mice, resulted in the formation of thick layers of lamellar bone and active osteoblasts that line the ceramic surface.

In a preclinical animal study, Bruder *et al.* showed the ability of autologous MSCs loaded into HA/TCP carriers to heal a critical-sized segmental defect in a canine model. Significant bone formation occurred at the host–implant interface, and a continuous span of bone was seen across the defect. Evidence of both woven and lamellar bone was seen. Periosteal calluses formed around the implant. During the 16-week period of the study, the callus remodeled and resulted in healing of the defect with bone that was similar in shape and size to the original segment of bone that was resected. Human MSCs loaded onto HA/TCP carriers were implanted into femoral defects in athymic rats in a study by the same group. Radiographic and histologic evidence of new bone formation was seen at eight weeks. Biomechanical testing showed that cell-loaded ceramic implants had more than twice the stiffness and torque to failure as ceramic implants that had no cells.

More recently, several groups have reported on the successful clinical application of tissue engineering strategies in the repair of bony defects in patients secondary to trauma and tumor resection. Quarto *et al.* demonstrated successful bony union in patients with segemental long bone defects, ranging from 4.0 to 7.0 cm, that were bridged with constructs consisting of osteoprogenitor cells loaded onto hydroxyapatite scaffolds. To avoid donor-site bone defect, a mandibular reconstruction was performed by

a group in Germany with the prefabrication of a custom-vascularized bone graft utilizing bone marrow aspirates, BMP-7, and a titanium mesh in a patient with prior ablative tumor surgery.

Growth factors in the bone morphogenetic protein (BMP) family, including BMP-2 and BMP-7, also induce progenitor cells to differentiate down the osteogenic lineage. Current strategies for bone tissue engineering include the use of osteoconductive matrix devices that promote bony ingrowth, and the delivery of osteoinductive growth factors to bony defect sites. Matrix materials include calcium HA, type I collagen gel, polylactic acid polymers, and demineralized bone matrix. Yasko *et al.* demonstrated histological and radiographical evidence of healing in segmental bone defects using demineralized bone matrices implanted with recombinant human bone morphogenetic protein-2 (*rhBMP-2*). In a study involving a larger animal model, a sheep femoral defect model was used by Gerhart *et al.* to show the efficacy of *BMP-2* in healing critical-sized long bone defects in a large animal model. A prospective, randomized trial of 450 patients looking at the safety and efficacy of *rhBMP-2* in improving the outcome of open tibial fractures showed that patients treated with intramedullary nailing and *rhBMP-2* had significantly lower risks of delayed union, and decreased need for more invasive interventions such as bone grafting and nail exchange. Moreover, patients tended to have significantly faster fracture healing, lower risk of infection, and less need for hardware removal. Gene therapy using genetically-manipulated MSCs is an attractive alternative method for delivery of growth factors. Not only can they safely deliver sustained release of growth factors to anatomic sites, but the osteogenic potential of MSCs also allows them to serve as substrates for osteoinductive factors and building blocks for newly-formed bone.

Regional gene therapy using MSCs as a vehicle for localized expression of osteoinductive proteins has shown promising results in animal models. Lieberman *et al.* showed that human bone marrow stromal cells transfected with adenoviral-*BMP-2* led to the formation of more robust, trabecular bone in an athymic rat femoral defect model, compared with the thin, lace-like bone that formed in defects filled with matrix carrying localized recombinant human *BMP-2* (*rhBMP-2*). Moreover, the femurs from the adenoviral *BMP-2* showed no statistically significant difference in biomechanical strength with respect to ultimate torque to failure and energy to failure, compared with control femurs. The difference in characteristics of the healing response may be related to the increased efficacy from sustained release of *BMP-2* from the adenovirally transfected bone marrow cells, compared with the single-dose response seen in the *rhBMP-2* group. Moreover, the implanted bone marrow stromal cells may themselves contribute to osteogenesis in the defect site. Dragoo *et al.* successfully transfected stem cells isolated from adipose tissue with the *BMP-2* gene, and showed rapid induction of the cells into the osteoblast phenotype in *in vitro* cell cultures. Collagen matrices seeded with the transduced cells were able to produce heterotopic bone in the hind limbs of SCID mice. Adipose tissue is plentiful and easily accessible, and may well serve as another alternative bone graft substitute in an orthopedic surgeon's armory.

Vascular invasion is a vital step in endochondral ossification. Vascular endothelial growth factor (VEGF) is one of the best-characterized angiogenic factors. Studies have also shown that it is essential during embryogenesis, skeletal development, and endothelial function. In their study, Peng *et al.* demonstrated that muscle-derived MSCs transduced with retroviral VEGF work synergistically with cells transfected with BMP-4 to enhance bone healing in critical-sized calvarial defects. VEGF was found to be important for endochondral bone formation through enhancement of angiogenesis, cell recruitment, improved cartilage formation, and accelerated cartilage resorption. This study demonstrated another strategy for use of stem cells in gene therapy-based treatment of skeletal defects.

Several applications of MSCs in the repair of local bone defects have been described previously. Bone marrow-derived MSCs may also hold great potential for treatment of systemic musculoskeletal disease. Osteoporosis and osteogenesis imperfecta (OI) are two of the more attractive candidates. OI is a genetic disorder of MSCs characterized by a defect in the type I collagen gene that results in children with growth retardation, short stature, and numerous fractures secondary to fragile bone. Six children have already been enrolled in a clinical trial at St Jude Children's Research Hospital that involves the intravenous administration of unmanipulated bone marrow from human leukocyte antigen (HLA)-identical or single-antigen-mismatched siblings. Five of the six patients showed engraftment in one or more sites, including bone, skin, and stroma. More importantly, these five patients all showed acceleration of growth velocity during the six-month postinfusion. The authors attributed the increase in growth to the generation of normal osteoblasts from the MSCs that engrafted in skeletal sites.

CARTILAGE

Osteoarthritis is one of the most prevalent chronic conditions in the United States, accounting for as many as 39 million physician visits a year. According to a National Health Interview Survey, it is estimated that approximately 70% of the population older than 65 years will have activity limitation, or require medical attention because of osteoarthritis. Moreover, a significant number of adolescents and young adults suffer from chondral defects secondary to trauma, sports-related injuries, and osteochondritis dissecans. Of 31 516 knee arthroscopies in a survey conducted

by Curl *et al.*, 63% of the knees had chondral lesions, with an average of 2.7 hyaline cartilage lesions per knee. Cartilage has poor intrinsic healing ability, and superficial defects typically do not heal spontaneously. This is related both to the lack of vascular supply, and to the poor proliferative ability of chondrocytes. When the lesion extends into the subchondral bone, mesenchymal cell recruitment occurs from the synovium and subchondral marrow, and a healing response ensues in which the defect is filled with repair tissue resembling fibrocartilage. Unfortunately, this tissue is structurally inferior to the native cartilage.

Articular cartilage is highly acellular, with cell volume averaging only approximately 2% of the total cartilage volume in adults. The extracellular matrix is composed of a highly-complex network of collagen fibrils and proteoglycans. Type II collagen is the dominant collagen subtype found in cartilage. The molecule is a triple helix, composed of three $\alpha 1$ chains and with multiple cross-links. The collagen fibrils contribute to the tensile strength of cartilage. Load-transmission capacity and compressive strength arise mainly from the proteoglycans, the other main constituent of the extracellular matrix of articular cartilage. The core protein of aggrecan contains a large number of chondroitin sulfate and keratin sulfate side chains that become highly hydrated. Aggrecan molecules also contain a hyaluronic acid-binding region.

Clinically, current treatment options for cartilage defects can be categorized into cartilage stimulation and cartilage replacement strategies. Cartilage stimulation techniques include abrasion arthroplasty, subchondral drilling, and the microfracture technique. However, the repair tissue never achieves the hyaline architecture of the native tissue. Osteochondral autografts and allografts represent the other spectrum where the defect is filled with plugs taken from normal regions of articular cartilage. The main disadvantages associated with use of autografts are paucity of tissue, donor-site morbidity, and the possiblity of long-term complications. Allografts, on the other hand, face the risk of donor rejection from immunogenicity, and disease transmission. Joint prosthesis remains the mainstay for symptomatic relief of pain and improvement of daily function of patients suffering from severe articular cartilage defects. In 1999 alone, more than 244 000 total knee arthroplasties were performed.

An exciting development has been the introduction of tissue engineering strategies for cartilage defects. Cell-based therapy for treatment of cartilage defects is already in use clinically, with the use of autologous chondrocytes. Chondrocytes are isolated and culture expanded from arthroscopically-harvested cartilage, and reimplanted into deep articular cartilage defects. Since 1987, more than 950 patients have been treated with this technique. Long-term (mean 7.5 years) follow-up of patients showed a good to excellent rating with the Cincinnati rating score. Biopsy specimens resembled hyaline cartilage with extracellular matrices that consisted primarily of type II collagen and aggrecan. Some of the disadvantages of this technology include a limited supply, and donor site morbidity associated with the initial cartilage harvest.

MSCs capable of chondrogenesis are present in bone marrow, periosteum, synovium, and adipose tissue. Human MSCs can be culture-expanded more than one-billion-fold, and retain their multi-lineage potential. Moreover, they proliferate rapidly, and have the advantage that they can recapitulate the embryonic events present in chondrogenesis. The importance of MSCs in cartilage repair is illustrated by the lack of a repair response in defects that do not penetrate subchondral bone. On penetration, recruitment of pluripotent marrow mesenchymal cells ensues. The influence of local cytokines from inflammatory cells and synovial fluid results in differentiation of the progenitor cells into chondrocytes and filling of the space with fibrocartilage.

The ability of MSCs to heal full-thickness articular cartilage defects was shown to be possible in a rabbit femoral condyle model. Wakitani *et al.* isolated bone marrow-derived and periosteum-derived MSCs from New Zealand White rabbits, and embedded them into a collagen-based scaffold. The cellular constructs were implanted into 6 mm long $\times$ 3 mm wide $\times$ 3 mm deep full-thickness cartilage defects in the weight-bearing portion of the medial femoral condyle. Healing of the defect by hyaline cartilage was evident as early as two weeks with filling of the subchondral space with dense, highly vascularized new bone. After four weeks, the neocartilage was thicker, and excellent integration was noted at the interface between the new subchondral bone and the host tissue. Moreover, further analysis of the histological specimen at the various time points showed that the formation of subchondral bone resulted from a recapitulation of the embryonic process of enchondral ossification, with progression of chondrocytes to a hypertrophic state, followed by vascular invasion and ossification. No evidence of osteoarthrosis was noted in any of the knees. At later time points, however, significant remodeling of the cartilage occurred, with loss of metachromatic staining. Genetic modification of the MSCs may enhance the healing response, and allow for better long-term repair tissue.

Mesenchymal stem cells were induced down the chondrogenic and osteogenic lineages separately, followed by a two-stage photopolymerization process with a polyethylene glycol-based hydrogel by Alhadlaq and Mao, to produce a tissue-engineered construct in the shape of an articular condyle. This has exciting implications in future treatment options in patients with osteoarthritis. Biological resurfacing of arthritic joints may soon become an alternative to total joint replacement. The feasibility of prefabrication of small joints and phalanges *in vivo* with tissue engineering techniques has also been demonstrated by others, with seeding of chondrocytes onto porous biodegradable scaffolds.

As we reach a better understanding of the biology and biomechanical properties of articular cartilage, and its

response to different growth factors, gene-modified tissue engineering can become a more powerful tool for cartilage resurfacing. A number of growth factors have been described that enhance chondrocyte proliferation and chondrogenic differentiation, including fibroblast growth factor-2 (FGF-2), insulin-like growth factor-I (IGF-I), TGFβ-1, growth hormone (GH), BMP-7 (also known as osteogenic protein-1), and BMP-2. Induction of chondrogenesis using growth factors can be accomplished by direct injection into the defect site, and application of gene therapy techniques.

With direct injections, single supraphysiological doses of growth factors may often be insufficient for proper healing. Degradation of the proteins may occur as a result of an inflammatory reaction, and the recruitment of macrophages and neutrophils. Moreover, dilution and clearance of growth factors by the surrounding synovial fluid occurs over time. Prolonged exposure to growth factors may be necessary for chondrogenic differentiation of resident progenitor cells. In addition, by using chondrocytes or MSCs as a delivery vehicle for growth factors via gene therapy, the cells can in turn be the building blocks for the regenerated cartilage, and respond to autocrine and paracrine growth factors. The newly-formed cartilage can be a chimera of the original cell construct and new mesenchymal progenitor cells, from the recruitment and migration that occurs from the actions of the cytokines secreted by the transduced cells. In a study by Grande *et al.* (1999), periosteal cells transfected with a retroviral vector containing *BMP-7* showed *in vitro* and *in vivo* gene expression for at least eight weeks after seeding onto polymer grafts. When the cell-based constructs were placed into 3 mm circular osteochondral defects in the intertrochlear grooves of rabbit knees, the defects were completely filled with predominantly hyaline-like tissue that persisted as along as 12 weeks after implantation.

Mandel *et al.* demonstrated the feasibility of successful transduction of bone marrow-derived mesenchymal progenitor cells, with both retroviral and adenoviral *TGFβ-1* vectors. These genetically modified progenitor cells were able to undergo chondrogenesis *in vitro*, with and without exogenous *TGFβ-1* addition. Nixon *et al.* constructed a replication-incompetent adenovirus vector expressing IGF-I, and successfully transfected chondrocytes, MSCs, and synoviocytes. Significant increases in proteoglycan production in the extracellular matrix secondary to *IGF-I* induction were noted. Moreover, cells expressed high levels of the *IGF-I* for up to 28 days in culture. Both of these studies verify the possibility of genetically-modifying MSCs so that they have the ability to secrete chondroinductive growth factors over sustained time periods.

Animal models for *ex vivo* gene therapy of articular cartilage defects using MSCs have already been carried out. Gelse *et al.* investigated the ability of adenoviral-mediated expression of perichondrial MSCs to heal an articular cartilage defect in a rat model. The effects of *AdBMP-2*- and *AdIGF-1*-transfected cells suspended in fibrin glue were evaluated with respect to their ability to heal the defects and integrate with the host tissue. The partial thickness lesions healed in both the *AdBMP-2* and *AdIGF-1* groups, with repair cartilage exhibiting hyaline morphology that was composed of type II collagen in the extracellular matrix, but no type I collagen. The defects in the nontransfected cell group primarily filled with fibrous tissue rich in type I collagen. Again, this is consistent with the observations seen in the natural repair process in which MSCs from marrow cavity progress to repair subchondral cartilage defects with fibrocartilage-like tissue. A complication of the *AdBMP-2* group that was not seen in the *AdIGF-1* group was osteophyte formation secondary to leakage of cells outside the construct.

Chondroprotective approaches to articular cartilage preservation have also been explored by several groups. Instead of trying to stimulate cartilage repair and replace cartilage defects, an alternative is prophylactic treatment and cessation of disease progression in its earlier stages. Interleukin-1 receptor antagonists (IL-1Ra), tumor necrosis factor (TNF) blockers, and interleukin-4 (IL-4) have all been reported to protect against degenerative changes in articular cartilage. The anti-inflammatory drugs infliximab (Remicade) and etanercept (Enbrel), both inhibitors of TNF, have been approved by the Food and Drug Administration for patients suffering from rheumatoid arthritis. Animal models have already shown the suppression of osteoarthritic changes, using gene therapy to effect delivery of IL-1Ra. Transduced MSCs expressing IL-1Ra or antagonists of TNF may both enhance the reparative process through their inherent chondrogenic potential, and retard the degradative process in cartilage lesions.

Avascular necrosis of the femoral head is a painful disorder of the hip that eventually leads to femoral head collapse, osteoarthritis, and the eventual need for a total hip replacement. In its early stages, treatment often consists of core decompression and various bone grafting techniques, including the use of vascularized fibular bone grafts. Recently, a group in Belgium demonstrated in a pilot study that percutaneous injection of mesenchymal stem cells into the zone of necrosis was effective in significantly reducing pain and joint symptoms, as well as delaying disease progression in patients. The authors postulated that the therapeutic effects of the injection are secondary to the introduction of progenitor cells to the region, as well as angiogenic growth factors from the bone marrow aspirates. Future strategies may incorporate the use of mesenchymal stem cells as delivery vehicles for chondrogenic and angiogenic growth factors.

MENISCUS

The annual incidence of meniscal tears is approximately 60 to 70 per 100 000. The menisci of the knee are semilunar

fibrocartilaginous structures that are integral to the normal function of the knee. The extracellular matrix consists of collagenous fibers that are mostly oriented circumferentially, with interspersing radial fibers that contribute to its structural integrity. The circumferential fibers help disperse compressive forces, while the radial fibers protect against tearing from tensile forces. The matrix contains mainly type I, II, and III collagens, with type I collagen being the most prevalent. The cells of the meniscus are fibrochondrocytes, because of their chondrocyte-like appearance and synthesis of fibrocartilaginous matrices. The main function of the meniscus is load transmission during weight-bearing. In extension, approximately 50% of the load in the knee is transmitted to the menisci. At 90º of flexion, this increases to almost 90% of the total load. The medial meniscus also acts as an important secondary restraint to anteroposterior translation, especially in the anterior cruciate ligament (ACL) deficient knee. Finally, the meniscus is important in shock absorption and joint lubrication.

Early in prenatal development, the menisci are very cellular and highly-vascularized. After skeletal maturity, the vascular zone is generally confined to the peripheral one-third of the meniscus. Longitudinal tears in the peripheral zone can generally be repaired, whereas radial tears are usually not amenable to repair. Defects that cannot be repaired are usually debrided to avoid the irritation from loose meniscal flaps. Total meniscectomy is usually contraindicated, because it leads to a number of osteoarthritic changes originally described by Fairbank. Long-term follow-up studies showed that a high percentage of patients with a history of meniscectomy go on to develop knee instability, and considerable amounts of radiographic degenerative changes in the knee.

Clearly, there is a need for new treatment options. Currently, meniscal allograft transplantation is one of the few alternatives for replacement of large meniscal defects. Unfortunately, there is a risk of rejection and disease transmission with allograft tissue. Moreover, the graft size of the donor must match the recipient, which can sometimes be difficult. Long-term problems with meniscal allografts include graft shrinkage, decreased cellularity, and loss of normal biological activity. Other experimental methods for meniscal repair include the use of fibrin clots, collagen-based polymers, and a number of polyurethane-based meniscal prostheses.

MSCs may provide novel treatment strategies both in growth-factor-based and cell-based treatment options for meniscal tears. A number of cytokines, including platelet-derived growth factor (PDGF), BMP-2, hepatocyte growth factor (HGF), epidermal growth factor (EGF), IGF-I, and endothelial cell growth factor, have been implicated in the proliferation and migration of meniscal cells within the different zones. Single doses of growth factors may not provide adequate stimulus in the repair tissue. Successful adenoviral-mediated expression of *BMP-2*, *PDGF*, *EGF*, and *IGF-1* has already been demonstrated in culture systems of MSCs and fibroblasts. MSCs can serve as delivery vehicles for sustained release of growth factors that contribute to cell proliferation, recruitment, and differentiation, and an increase in localized vascularization. Martinek *et al.* demonstrated the feasibility of gene therapy in tissue-engineered meniscal tissue, by showing evidence of transduced gene expression in meniscal allografts at four weeks. In another study by Hidaka *et al.*, bovine meniscal cells were transfected with an adenovirus vector encoding *HGF*, seeded onto polyglycolic acid scaffolds, and placed into subcutaneous pouches of athymic mice. Gene expression of *HGF* was associated with a significant increase in neovascularization.

The repair of meniscal defects by fibrocartilage has been advocated by some authors. Lesions in the avascular zone of canine menisci repaired by implantation of polyurethane polymers showed evidence of fibrocartilaginous tissue, compared with only fibrous tissue in the control group. In a small clinical series, Rodkey *et al.* implanted resorbable collagen scaffolds into medial meniscus lesions, and showed preservation of the joint surfaces and evidence of regeneration of tissue regeneration two years postoperatively. The chondrogenic potential of bone marrow-derived MSCs has been well-characterized. An alternative strategy in meniscal repair would be a prefabrication of a cartilage-like construct, using stem cells and a biodegradable matrix. Data from a rabbit partial meniscectomy model showed that MSCs embedded in a collagen sponge can enhance the formation of fibrocartilage tissue at the defect site. Although the tissue would not have identical properties of the native menisci, it would serve a similar function in joint surface preservation and load transmission across the knee joint. In a caprine model by Murphy *et al.*, osteoarthritis was induced by medial meniscectomy and ACL resection. At six weeks, an intra-articular injection of 10 million autologous mesenchymal stem cells was effective in leading to marked regeneration of the medial meniscus, and retarding the progression of osteoarthritis, compared to the control group.

LIGAMENTS AND TENDONS

Ligaments and tendons are bands of dense connective tissue that lend stability and provide movement of joints. Injury that leads to inflammation or tear of these structures can result in significant functional deficits, and development of degenerative joint disease. During the normal healing process, various growth factors, such as PDGF, FGF, and TGFβ are released by macrophages and platelets, to stimulate fibroblast proliferation and tissue remodeling. These growth factors contribute to proliferation, extracellular matrix secretion, and recruitment of cells. Tissue-engineered cellular

constructs and delivery of growth factors via gene therapy offers great potential in augmenting the healing process.

In general, most of the dry weight of skeletal ligaments is made up of collagen. Greater than 90% of this is type I, with a small percentage of type III. Glycosaminoglycans and elastin also comprise a small proportion of the biochemical make-up. Ligaments are oriented to resist tensile forces along their long axes. The diameter of collagen fibril diameters and the number of collagen pyridinoline cross-links correlate with the tensile strength of the healed ligament. Ligaments work in conjunction with muscle-tendon forces, bony intra-articular constraints, and other soft tissues to help stabilize joints and prevent nonphysiological movements. There are differences in the intrinsic healing capacity of various ligaments. After injury, ligaments heal in a series of stages: hemorrhagic, inflammatory, proliferation, and remodeling. Clinically, medial collateral ligament (MCL) injuries heal reliably without surgical intervention, whereas ACL tears usually do not heal spontaneously. Histological studies of human ACL tears show that, unlike extrasynovial ligaments, no evidence of bridging occurs between remnants of the ACL from the femoral and tibial sides. Typically, ACL ruptures are treated with a variety of different tendon autografts and allografts. Unfortunately, autograft harvest involves damage to previously healthy tissue, and allografts carry the risk of disease transmission. Moreover, although isolated MCL tears do well with nonoperative treatment, biochemical analysis shows that untreated MCL scar has a higher than normal proportion of type III collagen, higher collagen turnover rates, and increases in total glycosaminoglycan. Histologically, the extracellular matrix never completely approaches the highly-organized appearance of normal ligament substance. Studies have also shown that MCL fibroblasts migrate more rapidly, and repopulate cell-free areas more rapidly, than those from ACLs. Structural differences exist between cells of the MCL and the ACL.

Growth factors play a large role in healing and remodeling of musculoskeletal tissue. Schmidt *et al.* showed exposure to EGF and basic FGF leads to a significantly higher rate of proliferation in fibroblasts from the MCL and ACL. Fibroblast proliferation is a major component of the normal ligament healing process. Similarly, Marui *et al.* showed that FGF and EGF treatment of ligament fibroblast cultures leads to increased collagen synthesis. IGF-I also stimulates type I collagen synthesis in fibroblasts. However, complex interactions exist between different growth factors, because some act synergistically, whereas others antagonize one another. The structural and functional properties of tissues are largely dependent on the composition of the extracellular matrix.

The use of MSCs in gene therapy strategies for ligamentous and tendon injuries has many potential clinical uses. Growth factors can be delivered locally to injury sites that can promote each of the four stages of normal healing. Increased fibroblast proliferation effected by FGF and EGF can be promoted through adenoviral-mediated gene expression. Short bursts of growth factors will enable the healing process to occur more rapidly in the initial stages. TGFβ-1 promotes wound healing in many animal models. Its ability to augment cellular proliferation and increase secretion of collagen in the extracellular matrix hold great promise as a useful target gene for regional growth factor delivery. Animal studies by Hildebrand *et al.* and Batten *et al.* have already shown that MCLs exposed to PDGF leads to stiffness and breaking energy similar to their respective controls. Moreover, Letson and Dahners showed that combination treatment with IGF-I and FGF further enhances these structural properties. In a canine model by Kobayashi *et al.*, basic FGF leads to increased neovascularization and better orientation of collagen fibers in a partially ruptured ACL. The feasibility of using cells as a delivery vehicle of growth factors into the knee joint was confirmed by a study by Bellicampi *et al.*, showing cell-seeded collagen scaffolds that remained viable for up to four weeks when implanted in the knee joint.

In addition to the use of autografts and allografts, current treatment options that have been proposed for tendon defects include use of synthetic polymers and acellular biodegradable scaffolds. Scaffolds serve as a conduit for recruitment of cells during the initial healing process. As they degrade over time, the mechanical loading forces are transferred to the new repair tissue. Despite the promising data regarding repair strength and biomechanical properties, the immunogenicity and biocompatibility of the materials over time are not well understood. It would be ideal to use autologous sources of cells, in combination with biodegradable matrices, as a tissue-engineered construct to reconstruct ligaments or tendons.

Fibroblasts generate tension and change their orientation along tensile forces when cultured in three-dimensional collagen gels. MSCs display a similar fibroblastic property. The combination of MSCs with biodegradable scaffolds could be useful for bridging large tendon defects. Autologous cell-based tendon repair was performed in a hen flexor tendon model by Cao *et al.*, in which tenocytes seeded onto polyglycolic acid scaffolds were able to heal a 3–4 cm defect. The healed tissue resembled native tendon grossly and histologically. Biomechanically, the experimental group had 83% of the breaking strength of the normal tendon. Similarly, MSC-based repairs of Achilles tendon in animal models showed improvement of the biomechanics and function of the tendon. However, the histological appearance and biomechanical strength are inferior to normal tendon controls.

In one study by Awad *et al.*, collagen gels were seeded with MSCs in a full-thickness patellar tendon defect model to compare its effectiveness with the natural repair process. At 26 weeks after the original implantation, composite constructs showed significantly higher moduli and maximum stresses, compared with the natural repair group. When

compared with native tissue, however, the maximum stress was only 25% of the control. Moreover, 28% of the cell–matrix constructs formed bone in the tendon repair site.

Young *et al.* seeded rabbit MSCs onto a pretensioned polyglyconate suture to create a contracted construct. At 40 hours, cell nuclei were spindle shaped, and a cell viability of approximately 75% was noted in the constructs. Repair tissue treated with MSCs had a significantly larger cross-sectional area at the repair site than the contralateral control (suture only) and untreated native tissue. Cell-seeded repairs showed superior biomechanical properties with a two-fold increase in load-related properties. Compared with native tissue, the treated tendons had almost two-thirds of the structural properties at 12 weeks. A rapid rate of increase in load-related material properties may reflect the remodeling stage of healing mediated by the MSCs. Histologically, increases in tenocytes and collagen crimping pattern also occurred over time. The two studies reviewed previously demonstrate the potential of MSCs to help augment the tendon repair process. Animal models with rabbit patellar tendon repairs and Achilles tendon defects have also revealed the enhancement of tendon healing and improvement in biomechanical properties with the introduction of mesenchymal stem cells to the repair tissue.

Tissue-engineered constructs combining stem cells with biodegradable scaffolds enhance the biomechanical properties of the repair tissue and remodels over time to more closely resemble the complex organization of normal tendon. Preincubation of the constructs *in vitro* with growth factors may produce better biologic substitutes. Moreover, transfection of MSCs with genes such as *TGFβ-1*, *EGF*, and *FGF* may lead to a synergistic effect and to stronger structural repair tissue. Successful transfection of myoblasts and ACL fibroblasts with adenovirus and subsequent introduction into the rabbit ACL has been demonstrated. Expression of the *lacZ* reporter gene was noted at seven days, and persisted as long as six weeks. *BMP-12* gene transfer into lacerated chicken tendon resulted in augmentation of ultimate force and stiffness of the repaired tissue. Lou *et al.* previously demonstrated that mesenchymal progenitor cells form tendon-like tissue ectopically when transfected with the *BMP-12* gene.

SPINE

Posterolateral lumbar intertransverse process fusions are commonly performed for spinal disorders secondary to degenerative changes and trauma. However, the nonunion rates have been reported to be as high as 40% with single-level fusions, and even higher in multiple-level procedures. Currently, nonunions are prevented by application of instrumentation, such as pedicle screws, rods or plates, and various interbody fusion devices, to achieve better correction of deformities and biomechanical stability during the healing period. However, a significant number of nonunions and pseudoarthrosis still exist in fusions with instrumentation, and some have shown no statistically significant decrease in nonunions with use of screw fixation.

More recently, a large number of clinical studies have compared the efficacy of osteoinductive proteins with autogenous iliac crest bone graft in lumbar fusions. Johnsson *et al.* showed, in a small randomized clinical series, that osteogenic protein-1 (OP-1), also known as BMP-7, was as effective as autogenous bone graft in achieving single-level lumbar fusions. In a rabbit model by Patel *et al.*, *BMP-7* was found to overcome the inhibitory effects of nicotine on spinal fusion. In a prospective, randomized, clinical trial, Boden *et al.* compared Texas Scottish Rite Hospital (TSRH) pedicle screw instrumentation with rhBMP-2, with TSRH pedicle screw instrumentation, and rhBMP-2 without instrumentation, and showed that the groups with rhBMP-2 had 100% fusion rates compared with only 40% (2 out of 5) fusion in the TSRH pedicle screw instrumentation-only group. Improvement of the Oswetry score at follow-up was highest in the rhBMP-2 only group. The use of *rhBMP-2* as an adjunct in fusion using lumbar interbody devices was demonstrated by Sandhu *et al.* in a sheep model. Recombinant BMP-2 was shown to be superior to autogenous bone graft. Minimally invasive spinal fusion and relief of discogenic back pain was possible in a series of 22 patients undergoing laparoscopic placement of *rhBMP-2*.

Direct introduction of growth factors into the intervertebral space for spinal fusion, however, does have its risks. Heterotopic bone formation could occur from leakage of osteoinductive proteins outside its carrier into the surrounding tissue. This could be devastating, especially in the setting of dural tears. Moreover, most osteoinductive growth factors are rapidly-metabolized so that single-bolus injections may not achieve ideal efficacy in clinical settings. Gene therapy using MSCs offers the ability to deliver sustained release of growth factors to a local site. Successful spinal fusion has been shown by Boden *et al.* in a rat study using bone marrow cells transfected with a novel osteoinductive protein, LIM mineralization protein-1 (*LMP-1*). LMP-1 is thought to be a soluble osteoinductive factor that induces expression of other BMPs and their receptors. Wang *et al.*, in an animal model of spinal fusion using adenovirus-mediated gene therapy, showed that radiographic evidence of fusion was present by four weeks postoperatively in rats treated with MSCs expressing BMP-2. Moreover, the group receiving *AdBMP-2*-transfected cells showed repair with coarse trabecular bone, compared with thin, lace-like bone in the rhBMP-2 group. The long-term sustained release of osteoinductive proteins was perhaps more effective in producing a stronger biological response than a single supraphysiological dose. Another possibility could be that the MSCs differentiated down the osteogenic lineage, and are now contributing to the new bone in the fusion mass.

Similar to cartilage repair strategies, another potential strategy in the treatment of spinal disorders would be the suppression of disc degeneration. Fraying, splitting, and loss of collagen fibers in the intervertebral discs and calcification of the cartilage in the endplates occur with age. Matrix metalloproteinases and aggrecanases are also thought to be important in extracellular matrix degradation in the disc. Proteoglycans are important for maintenance of disc height and its compressive ability, and their preservation is essential for the load-bearing capacity of intervertebral discs. Thompson *et al.* studied the effect of various growth factors on proliferation and proteoglycan synthesis of annular pulposus and nuclear pulposus cells isolated from canine intervertebral discs, and found that TGFβ-1 and EGF induced a five-fold increase in proteoglycan production. Retardation of programmed cell death in annulus cells, and a dose-dependent increase in proteoglycan synthesis, has been shown in discs exposed to IGF-1. Delivery of *TGFβ-1* and/or *IGF-I* to intervertebral discs using MSCs as a vehicle for gene therapy, therefore, represent two possible strategies in the treatment of disc disorders.

Using a hyaluronan gel as a carrier, mesenchymal stem cells were successfully introduced inside the intervertebral discs in a rat model. Mesenchymal stem cells were found to stay viable and proliferate *in situ* with the disc. In a rabbit model, Sakai *et al.* demonstrated the ability of transplanted mesenchymal stem cells to delay disc degeneration and maintain disc integrity. Successful retroviral-mediated gene transfer of *lacZ* and *IL-1Ra* of cultured chondrocytes has been performed by Wehling *et al.* The authors suggested the clinical feasibility of harvesting endplate tissue cartilage from patients with degenerative discs and the subsequent reintroduction of genetically modified cells into the disc space. This same strategy can be applied using MSCs that can be isolated easily from the iliac crest. As mentioned previously, adenoviral-mediated expression of *IGF-I* by MSCs has already been successfully shown. Gruber *et al.* (2003) showed the efficacy of *in vivo TGFβ-1* gene transfer to intervertebral discs, and its ability to increase the level of proteoglycan synthesis.

SUMMARY

MSCs hold great potential for the development of new treatment strategies for a host of orthopedic conditions. Animal models have demonstrated the wide spectrum of clinical situations in which MSCs could have therapeutic effects. Clinical application of the principles of cell-based tissue engineering is already seen with the Carticel (Genzyme, Cambridge, Massachusetts) program that uses culture-expanded autologous chondrocytes in the repair of articular cartilage defect. The multi-lineage potential and plasticity of MSCs allow them to be building blocks for a host of nonhematopoietic tissues, including bone, cartilage, tendon, and ligament. Advances in fabrication of biodegradable scaffolds that serve as beds for MSC implantation will hopefully lead to better biocompatibility and host tissue integration. Minimal toxicity has been observed in animal models involving genetically-manipulated stem cells transduced with retroviral and adenoviral vectors. Gene therapy using stem cells as delivery vehicles is a powerful weapon that can be used in a plethora of clinical situations that would benefit from the osteoinductive, chondroinductive, proliferative, and angiogenic effects of growth factors.

KEY WORDS

Gene therapy Novel strategy in treating diseases by modifying the expression of specific genes toward a therapeutic goal.

Osteoconductive Scaffolds or materials that are permissive to bone formation, but do not attract osteoprogenitors that initiate bone formation.

Osteoinductive Containing growth factors that signal attraction of osteoprogenitors that lead to osteoblastic differentiation and bone formation.

Pluripotent stem cells Cells not capable of growing into a whole organism, but able to differentiate into any cell type in the body.

Tissue engineering The development of biological substitutes for regeneration and/or repair of defective tissue by combining cells with appropriate bioactive scaffolds in combination with differentiation stimuli.

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Chapter 63

Embryonic Stem Cells in Tissue Engineering

Shulamit Levenberg, Ali Khademhosseini, and Robert Langer

Tissue engineering is an interdisciplinary science that involves the use of biological sciences and engineering to develop tissues that restore, maintain, or enhance tissue function. However, to realize the dream of creating off-the-shelf organs for transplantation, a renewable source of cells is required. Embryonic stem (ES) cells have the potential to provide such a source of cells for tissue engineering applications, because of their ability to differentiate to all somatic cells, and their unlimited proliferative capability. However, to use ES cells in tissue engineering a number of challenges must be addressed regarding methods to direct ES cell differentiation, overcome host's immune rejection, prevent tumor formation, scale-up the production process, and control scaffold properties better. This chapter will introduce tissue engineering approaches, and the role of ES cells in various tissue engineering applications.

INTRODUCTION

Traditionally, approaches to restore tissue function have involved organ donation. However, despite attempts to encourage organ donations (Platt, 1998; Niklason and Langer, 2001), there is a shortage of transplantable human tissues such as bone marrow, hearts, kidneys, livers, and pancreases. Currently, more than 74 000 patients in the United States are awaiting organ transplantation, while only 21 000 people receive transplants annually (Petit-Zeman, 2001).

Tissue engineering-based therapies may provide a possible solution to alleviate the current shortage of organ donors. In tissue engineering, biological and engineering principles are combined to produce cell-based substitutes, with or without the use of materials. One of the major obstacles in engineering tissue constructs for clinical use is the limit of available human cells. Stem cells isolated from adults or developing embryos are a current source for cells for tissue engineering. The derivation of human embryonic stem (hES) cells in 1998 (Shamblott *et al.*, 1998; Thomson *et al.*, 1998), has generated great interest in their potential application in tissue engineering. This is because of the ability of ES cells to grow in culture, and to give rise to differentiated cells of all adult tissues. However, despite their therapeutic potential, both adult and ES cells present a number of challenges associated with their clinical application. For example, although adult stem cells can be directly isolated from the patient, and are therefore immunologically compatible with the patient, they are typically hard to isolate and grow in culture. In contrast, ES cells can be easily grown in culture and differentiated to a variety of cell types, but ES-derived cells may be rejected by the patient, and undifferentiated ES cells may form tumors.

The goal of this chapter is to analyze the potential of ES cells in tissue engineering. The chapter discusses the importance of ES cells as a source of cells for tissue engineering by using examples from the current research in the field. Furthermore, the chapter discusses some of the fundamental principles and seminal work in tissue engineering.

TISSUE ENGINEERING PRINCIPLES AND PERSPECTIVES

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to develop biological substitutes, typically composed of biological and synthetic components, which restore, maintain, or improve tissue function (Nerem, 1991; Langer and Vacanti, 1993). Tissue-engineered products would provide a lifelong therapy, and would greatly reduce the hospitalization and healthcare costs associated with drug therapy, while simultaneously enhancing the patients' quality of life.

In general there are three main approaches to tissue engineering (Langer and Vacanti, 1993):

1. to use isolated cells or cell substitutes as cellular replacement parts;

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2. to use acellular materials capable of inducing tissue regeneration;
3. to use a combination of cells and materials (typically in the form of scaffolds).

Although host stem cells could be involved in all of these approaches, ES cells can be directly involved in the first and the third approach.

Isolated Cells or Cell Substitutes as Cellular Replacement Parts

Isolated cells have been used as a substitute for cell replacement parts for many years. In fact, the first application of stem cells as a cellular replacement therapy is associated with bone marrow transplantation or blood transfusion studies, in which donor hematopoietic stem cells repopulated the host's blood cells (Till and McCulloch, 1980). Other stem cells have demonstrated their potential in various diseases. For example, bone marrow-derived cells have been shown to: (1) give rise to endothelial progenitor cells that were used to induce neovascularization of ischemic tissues (Kocher *et al.*, 2001; Edelberg *et al.*, 2002; Luttun *et al.*, 2002; Walter and Dimmeler, 2002); (2) to regenerate myocardium (Orlic *et al.*, 2001); (3) to give rise to bone, cartilage and muscle cells (Pittenger *et al.*, 1999); (4) migrate into the brain to give rise to neurons (Brazelton *et al.*, 2000; Mezey *et al.*, 2000). In addition, myoblasts isolated from skeletal muscle that on injection into the heart restored heart muscle function (Dorfman *et al.*, 1998; Taylor *et al.*, 1998), and neural stem cells that resulted in the treatment of Parkinson's disease (Fricker, 1999; Okano *et al.*, 2002; Storch and Schwarz, 2002), are some examples of other potential adult stem cell-based therapies. Tissue engineering products based on cells have been developed in the form of skin substitutes through the use of allogeneic cells (from companies such as Organogenesis and Advanced Tissue Sciences). In addition, the injection of mesenchymal stem cells is underway for cartilage and bone regeneration (Bruder *et al.*, 1998).

Embryonic stem cells provide an alternative source of cells for cellular substitutes. *In vitro* ES cells have been shown to give rise to cells of hematopoietic (Bigas *et al.*, 1995; Palacios *et al.*, 1995; Kaufman *et al.*, 2001), endothelial (Hirashima *et al.*, 1999), cardiac (Rohwedel *et al.*, 1994; Fleischmann *et al.*, 1998; Kehat *et al.*, 2001), neural (Brustle *et al.*, 1999; Reubinoff *et al.*, 2001; Schuldiner *et al.*, 2001; Zhang *et al.*, 2001), osteogenic (Buttery *et al.*, 2001), hepatic (Hamazaki *et al.*, 2001), and pancreatic (Assady *et al.*, 2001; Lumelsky *et al.*, 2001) tissues. Although ES cells provide a versatile source of cells for generation of many cell types, so far only a few experiments have demonstrated the use of ES cells to replace functional loss of particular tissues. One such example is the creation of dopamine-producing cells in animal models of Parkinson's disease (Bjorklund *et al.*, 2002; Kim *et al.*, 2002). These ES cell derived highly-enriched populations of midbrain neural stem cells generating neurons that showed electrophysiological and behavioral properties similar to neurons. Although functional properties of neurons derived from human ES cells still need to be investigated, it has been shown that human ES cells derived neural precursors can be incorporated into various regions of the mouse brain, and differentiate into neurons and astrocytes (Zhang *et al.*, 2001). Also, human ES cells that were differentiated to neural precursors were shown to migrate within the host brain, and differentiate in a region-specific manner (Reubinoff *et al.*, 2001). ES cells were also tested for future use in the heart. It was shown that mouse ES-derived cardiomyocytes were morphologically similar to neighboring host cardiomyocytes (Klug *et al.*, 1996; Kehat *et al.*, 2001, 2002; Min *et al.*, 2002; Yang *et al.*, 2002; Mummery *et al.*, 2003). In addition, mouse ES cells that were transfected with an insulin promoter (driving expression of the neo gene, a marker for antibiotic resistance), have been shown to give rise to insulin-producing cells that can restore glucose levels in animals (Soria *et al.*, 2000). Although these functional data were obtained by using genetically-modified ES cells, insulin production from ES cells (Assady *et al.*, 2001; Lumelsky *et al.*, 2001) suggests that these cells may potentially be used for the treatment of diabetes.

ES cells have also been shown to give rise to functional vascular tissue. Early endothelial progenitor cells, isolated from differentiating mouse ES cells, were shown to give rise to three blood vessel cell components, hematopoietic, endothelial, and smooth muscle cells (Yamashita *et al.*, 2000). Once injected into chick embryos these endothelial progenitors differentiated into endothelial and mural cells, and contributed to vascular development. We have shown that human ES cells can differentiate into endothelial cells, and isolated these cells using platelet endothelial cell adhesion molecule-1 antibodies. *In vivo*, when transplanted into immunodeficient mice, the cells appeared to form microvessels (Levenberg *et al.*, 2002).

Using a Combination of Cells and Materials

Tissue engineering approaches that use cells and scaffolds can be categorized into two categories; open and closed systems (Uludag *et al.*, 2000). These systems are distinguished based on the exposure of the cells to the immune system on implantation.

Open Systems

In open tissue engineering systems, cells are immobilized within a highly-porous, three-dimensional scaffold. The

scaffold could either be comprised of synthetic or natural materials, or composites of both (Langer and Vacanti, 1993; Lanza *et al.*, 1997; Langer and Vacanti, 1999; Vacanti and Langer, 1999). Ideally, this scaffold provides the cells with a suitable growth environment, optimum oxygen and nutrient transport properties, good mechanical integrity, and a suitable degradation rate. The use of scaffolds provides three-dimensional environments, and brings the cells in close proximity so that it provides the cells with sufficient time to enable self-assembly and formation of various components that are associated with the tissue microenvironment. Ideally the material is degraded as cells deposit their extracellular matrix molecules. The materials used for tissue engineering are either synthetic biodegradable materials, such as hydroxyapatite (Laurencin *et al.*, 1996; Friedman and Costantino, 1998; Pelissier *et al.*, 2003), calcium carbonate (Pelissier *et al.*, 2003; LeGeros, 2002; Li *et al.*, 2002), poly(lactic acid) (PLA) (Laurencin *et al.*, 1996; Mooney *et al.*, 1996), poly(glycolic acid) (PGA) (Mooney *et al.*, 1996), poly lactic-glycolic acid (PLGA), poly(propylene fumarate), and polyarylates, or natural materials such as collagen (Chevallay and Herbage, 2000) and alginate (Rowley *et al.*, 1999). Natural materials are typically more favorable to cell adherence, while the properties of synthetic materials, such as degradation rate, mechanical properties, structure, and porosity, can be better controlled (Langer and Vacanti, 1993).

Open tissue engineering systems have been successfully used to create a number of biological substitutes, such as bone (Cancedda *et al.*, 2003; Ohgushi *et al.*, 2003), cartilage (Rahman and Tsuchiya, 2001; Baek *et al.*, 2002; Sherwood *et al.*, 2002), blood vessels (Niklason and Langer, 1997; Niklason *et al.*, 1999), cardiac (Shinoka *et al.*, 1998), smooth muscle (Kim *et al.*, 1999), pancreatic (Cui *et al.*, 2001), liver (Hasirci *et al.*, 2001), tooth (Young *et al.*, 2002), retina (Lu *et al.*, 2001), and skin (Herson *et al.*, 2001; Badiavas *et al.*, 2002) tissues. Several tissue-engineered products are under clinical trials for Food and Drug Administration (FDA) approval. Engineered skin or wound dressing and cartilage are two of the most advanced areas with regard to clinical potential (Niklason and Langer, 2001). For example, a skin substitute that consists of living human dermis cells in a natural scaffold consisting of type I collagen already received FDA approval for use in a diabetic foot ulcer (Griffith and Naughton 2002). In addition, various cartilage and bone products are currently also in clinical stages, and bladder and urologic tissue are being tested in various stages of research (Oberpenning *et al.*, 1999).

Despite the ability of stem cells to differentiate into cells with the phenotypic and morphological structure of desired cell types, there have been very few scaffold-based tissue engineering studies that use ES cells. In the case of adult stem cells, scaffolds have been utilized in conjunction with mesenchymal stem cells (Boo *et al.*, 2002; Howard *et al.*, 2002; Weber *et al.*, 2002; Bensaid *et al.*, 2003; Yamada *et al.*, 2003), neural stem cells (Park *et al.*, 2002), and oval cells (Suh *et al.*, 2003). One such example is the transplantation of neural stem cells onto a polymer scaffold that was subsequently implanted into the infarction cavities of mouse brains injured by hypoxia-ischemia. These stem cells generated an intricate meshwork of many neurites, and integrated with the host (Park *et al.*, 2002). We have seeded neural stem cells onto specialized scaffolds (Lavik *et al.*, 2001, 2002), and have demonstrated spinal cord regeneration and improved hind-leg function of adult rats from a hemisection injury model (Teng *et al.*, 2002). Also, MSCs have been differentiated on polyethylene glycol (PEG) or PLGA scaffolds, and have been shown to give rise to cartilage or bone, depending on the medium conditions (Martin *et al.*, 2001).

ES cells may be differentiated in culture, desired cell types selected and subsequently seeded onto scaffolds. We have used this technique to study the behavior of ES cell-derived endothelial cells in tissue engineering constructs (Levenberg *et al.*, 2002). Human ES cell-derived endothelial progenitors that were seeded onto highly porous PLLA/PLGA biodegradable polymer scaffolds formed blood vessels that appeared to merge with the host vasculature when implanted into immunodeficient mice (Figure 63-1).

There may be other approaches to using ES cells or their progeny with scaffold-based tissue engineering systems. For example, it may be possible to differentiate ES cells directly on scaffolds in culture. Finally, it may be possible to differentiate genetically-engineered ES cells seeded onto scaffolds *in vivo* (see Figure 63-2).

Coercing cells to form tissues while differentiating is an important issue that has not been widely explored. This may be achieved by seeding ES cells directly onto the scaffolds, followed by inducing their differentiation *in situ*. Porous biodegradable polymer scaffolds can be used to support the ES cells, and they represent a promising system for allowing formation of complex 3D tissues during differentiation. The scaffold provides physical cues for cell orientation and spreading, and pores provide space for remodeling of tissue structures. These scaffolds should ideally provide the cells with cues to guide their differentiation into the desired cell types. The possible advantages of this system could involve the assembly of the cells as they differentiate. This differentiation pattern may mimic the developmental differentiation of the cells much more closely, and therefore may induce differentiation into the desired tissue. Ultimately, *in vitro* differentiated constructs can potentially be used directly for transplantation.

An approach which has not been considered as an alternative to *in vitro* differentiation of ES cells is to use the adult body's microenvironment to induce the differentiation of ES cells. *In vivo* differentiation of ES cells is not yet a feasible option, due to the tumorigenic nature of ES cells, as well as the heterogeneous cell population that results from

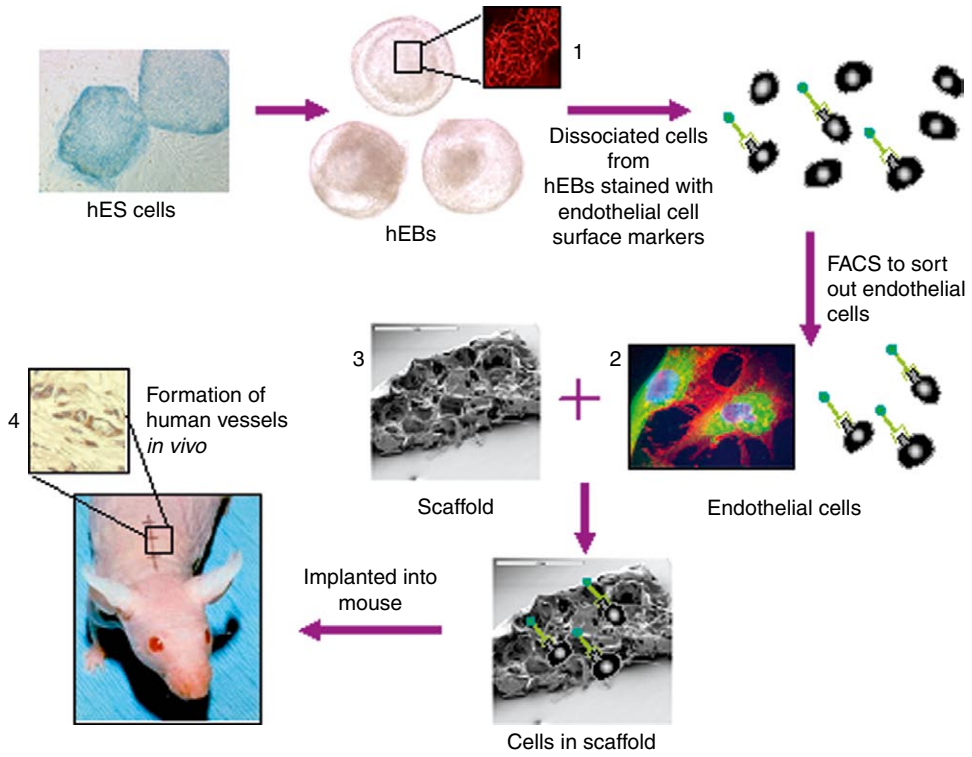


FIGURE 63-1 Approaches for using ES cell for scaffold based tissue engineering applications. ES cells can be used in tissue engineering constructs in a variety of methods. ES cells can be expanded in culture and then seeded directly onto scaffold where they are allowed to differentiate. Alternatively, stem cells can be directed to differentiate into various tissues and enriched for desired cells prior to seeding the cells onto scaffolds.

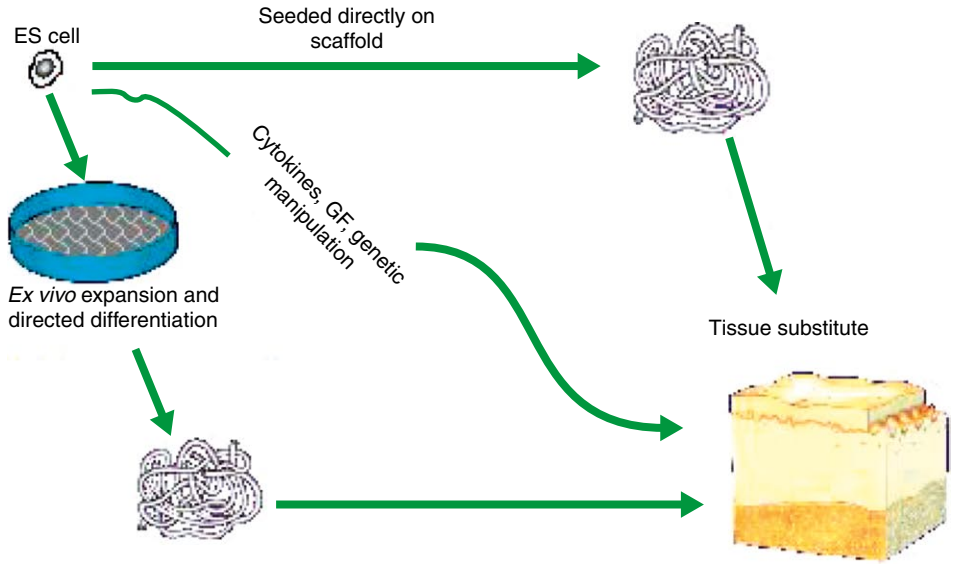


FIGURE 63-2 Embryonic endothelial cells on scaffolds *in vivo*. hES cells were induced to form EBs, in which differentiation into endothelial cells and formation of vessel-like network was observed (1). Embryonic endothelial cells were isolated from hEBs by staining dissociated EB cells with endothelial surface marker and sorting out positive cells using flow cytometry (FACS). Isolated endothelial cells (2) were seeded on polymer scaffolds (3) and implanted into immunodeficient mice. The embryonic endothelial cells appeared to form vessels *in vivo* (4). Confocal image of vessel network formation within 13-day-old hEB, stained with PECAM-1 antibodies. Isolated embryonic endothelial cells grown in culture stained with PECAM-1 (red) and VWF (green) antibodies. Scanning electron microscopy (SEM) of PLLA/PLGA scaffolds. Immunoperoxidase (brown) staining of seven day implants with anti-human PECAM1 antibodies showing vessels lined with human endothelial cells.

their non-directed differentiation. However, it may be possible to use a cell's apoptotic response mechanism to induce selective pressure for the desired cells *in vivo*. Thus, genetically-modified ES cells that undergo apoptosis on differentiation into undesirable cell types could be used to direct the differentiation of these cells, while similar approaches could be adopted to control their proliferative behavior.

Closed Systems

One of the main difficulties associated with open tissue engineering systems is the potential immunological issues associated with the implanted cells. Closed systems aim to overcome this difficulty by immobilizing cells within polymeric matrices that provide a barrier for the immunological components of the host. For example, cells can be immobilized within semi-permeable membranes that are permeable to nutrients and oxygen, while providing a barrier to immune cells, antibodies, and other components of the immune system (Lim and Sun, 1980; Uludag *et al.*, 2000). Furthermore, the implants can either be implanted into the patient, or used as extra-corpical devices. Closed tissue engineering systems have been used particularly for the treatment of diabetes (Chicheportiche and Reach, 1988; Zekorn *et al.*, 1992; Sefton *et al.*, 2000), liver failure (Uludag and Sefton, 1993; Chandy *et al.*, 1999; Wang *et al.*, 2000), and Parkinson's disease (Aebischer *et al.*, 1991; Esposito *et al.*, 1996; Wang *et al.*, 1997; Vallbacka *et al.*, 2001). This system may prove to be particularly useful in conjunction with ES cells, since the immobilization of ES cells within a closed system may overcome the immunological barrier that faces ES cell-based therapies. For example, ES cell derived β-cells that can response to insulin, or domaine-producing neurons can be used in clinics without fear of rejection. In addition, closed systems protect the host against potentially tumorigenic cells, as it limits the cells within the polymeric barrier. Currently, engineering and biological limitations, such as material biocompatibility, molecular weight cut-off, and the immune system's reaction to shed antigens by the transplanted cells, are some of the challenges that prevent these systems from widespread clinical applications.

LIMITATIONS AND HURDLES OF USING EMBRYONIC STEM CELLS IN TISSUE ENGINEERING

Despite significant progress in the field of tissue engineering, there are a number of challenges that are limiting the use of ES cells in tissue engineering. These challenges range from understanding stem cell biology questions, to how to control stem cell fate, to engineering challenges on scale-up, to business questions of feasibility and pricing.

Directing the Differentiation of Embryonic Stem Cells

Perhaps the biggest challenge in using ES cells in clinical applications is the lack of knowledge of how to direct their differentiation ability. All studies that have shown the generation of specific cell types have not shown a uniform differentiation into a particular cell type. This may be attributed to the intrinsic property of ES cells to differentiate stochastically in the absence of proper temporal and spatial signals from the surrounding microenvironment.

Techniques can be used to increase the ratio of cells that give rise to the desired lineages include genetic and microenvironmental manipulations. Genetic techniques can be categorized into positive or negative regulators. The positive regulators include the constitutive or controlled expression of transcription factors that have been shown to drive the differentiation into particular tissues. For example, the overexpression of Nurr transcription factor has been shown to increase the frequency of ES cells that differentiate into functional neural cells (Kim *et al.*, 2002). Alternatively, the negative regulators could be incorporated to induce the apoptosis of cells that differentiate to varying pathways. For example, neomycin selection and suicide genes that are activated by certain transcription factors can be used (Soria *et al.*, 2000). Clearly, these techniques will benefit from further understanding of the inner workings of transient cells, and knowledge of the differentiation pathways and lineages. Further analysis into stem cell and progenitor hierarchy through high-throughput analysis of microarray or proteomics data should accelerate this process (Phillips *et al.*, 2000; Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002).

Another important criterion is the functionality of ES cell-derived cells as a source of tissues. The importance of rigorous testing has become clear in studies in which nestin-positive putative pancreatic cells stained positive for insulin using antibodies, due to cellular uptake from the surrounding medium (Rajagopal *et al.*, 2003). Thus, the incorporation of protein and functional tests should accompany the morphological and phenotypic analysis that is often used in ES cell literature to characterize differentiated cells.

Isolating the Desired Cell Types for Therapy

One of the main problems with ES cell-based therapies is finding suitable techniques to isolate the desired cells from a heterogeneous population of cells. One approach is to allow for the random differentiation of the ES cells, followed by isolation using a cell surface marker. We have used this method for isolation of ES cell-derived endothelial cells using PECAM-1 receptor (Levenberg *et al.*, 2002). Also, ES cell-derived hematopoietic progenitors

have been isolated in a similar manner using CD34 marker (Kaufman *et al.*, 2001). Another potential method is through reporter gene knockin modifications. These modifications have already been used on ES cells to allow for labeling of cells at various stages of differentiation (Eiges *et al.*, 2001; Zwaka and Thomson, 2003). The utility of other techniques, such as magnetic separation, or use of neomycin selection, must be further examined for selecting various ES cell derived progeny.

Scale-up of Embryonic Stem Cells in Tissue Engineering

Although laboratory scale ES cell cultures have been shown to produce differentiated progeny for both rodent (Chen *et al.*, 2000) and human (Itskovitz-Eldor *et al.*, 2000) ES cells, it is generally accepted that these culturing methods are not feasible for large-scale production of ES cells for therapeutic applications. Production of a sufficient quantity of differentiated cells from ES cell is an important challenge in realizing the clinical potential of ES cells. The large-scale production of ES cells will likely be specific to the type of tissue being generated, and must remain reproducible, sterile, and economically feasible. Furthermore, this scale-up process must maintain the appropriate control over bioprocess conditions, such as mechanical stimuli, medium conditions, and physicochemical parameters (such as temperature, oxygen, pH, and carbon dioxide levels), as well as growth factor and cytokine concentrations.

ES cell differentiation protocols have generally used two-dimensional cultures and/or embryoid bodies. Although each technique provides specific advantages, differentiation of ES cells in embryoid bodies produces a wider spectrum of cell types (Magyar *et al.*, 2001). This has been attributed to the embryoid bodies' ability to better mimic the temporal pattern of cell differentiation as seen in the embryo. However, in some applications, the combined use of EBs and adherent cultures has resulted in better cell yields. For example, to induce ES cells to differentiate to cardiomyocytes, an EB formation in suspension cultures followed by a differentiation in adhesion cultures has been shown to optimize the percentage of cells that give rise to cardiomyocytes (Guan *et al.*, 1999; Klinz *et al.*, 1999). Similarly, the production of hepatocytes has been shown to be induced by first culturing the cell in EBs, followed by culturing on two-dimensional cultures (Hamazaki *et al.*, 2001).

The formation of EBs in laboratories has generally been performed using techniques that have not been ideal for large-scale production. For example, many studies have used the "hanging drop protocol" (Maltsev *et al.*, 1999), in which ES cells are placed within a "hanging drop," and allowed to form an aggregate which can then be differentiated. Other techniques have formed EBs by placing the cells on non-adherent tissue culture dishes, which once more place a limit on the quantity of cells produced. A technique which may allow for the scale-up of embryoid body cultures is the use of suspension cultures using spinner flasks (Dang *et al.*, 2002). Such cultures have been shown to enhance the supply of oxygen and nutrients to the cells within the embryoid body, by exposing the surface of the cell aggregate to continuous supply of fresh medium (Wartenberg *et al.*, 1998; Dang *et al.*, 2002).

To prevent the difficulties associated with EB heterogeneity, EBs have been immobilized in alginate microbeads. The microencapsulation of cells within these microbeads resulted in differentiation of cells into cardiomyocytes and smooth muscle cells (Magyar *et al.*, 2001). In addition, ES cells may adhere to beads with desired extracellular matrices and be differentiated. This approach also enhances the transport of medium and oxygen to the cells, in comparison to two-dimensional cultures, and provides additional mechanical stimuli which may be an improved alternative to two-dimensional culture systems.

To enhance the supply of medium to tissue-engineered scaffolds or embryoid bodies, methods other than passive diffusion may be required, such as the use of perfusion systems, in which the medium is flowed through the scaffold. Perfusion bioreactors have already been developed for a variety of tissue-engineering applications, such as cartilage and cardiac (Carrier *et al.*, 2002; Davisson *et al.*, 2002). For example, perfusion through scaffolds has been generated in rotating wall vessels (Freed *et al.*, 1997; Margolis *et al.*, 1999; Vunjak-Novakovic *et al.*, 1999; Martin *et al.*, 2000; Botchwey *et al.*, 2001), or through pumping the medium directly through the scaffolds (Davisson *et al.*, 2002) to grow chondrocytes for cartilage generation.

It is known that mechanical forces affect the differentiation and functional properties of many cell types (Niklason *et al.*, 1999; Vunjak-Novakovic *et al.*, 1999; Li and Xu, 2000; Martin *et al.*, 2000), therefore, ES cell based cultures that aim to direct the differentiation of ES cells require proper mechanical stimuli for the tissue. Understanding the effects of mechanical stimuli on ES cell differentiation is still primitive, but tissue-engineering systems have been developed that incorporate the effects of mechanical forces. For example, functional autologous arteries have been cultured using pulsatile perfusion bioreactors (Niklason *et al.*, 1999). Thus, the use of mechanical stimuli may further enhance the ability of these cells to respond to exogenous signals. Other environmental factors that may be required are the use of electrical signals and spatially regulated signals, to induce the differentiation and allow for maturation of the desired tissues. Hopefully, with time, such techniques will become particularly important in allowing for scaled-up ES cell based tissue-engineering applications. The development of bioreactors that control the spatial and temporal signaling that induces ES cell differentiation requires collaborative effort between engineers and biologists, and is currently in the early stages of its development.

Tissue Engineering Limitations

Synthetic scaffolds that support tissue growth by serving as the extracellular matrix for the cells do not represent the natural ECM associated with each cell type and tissue. ES cells and their progeny reside in a dynamic environment during development; thus, synthetic or natural substrates that aim to mimic the developing embryo must present similar signaling and structural elements. A number of approaches are currently under development which may prove useful for ES cells. The use of "smart" scaffolds that release particular factors (Murphy *et al.*, 2000) and/or control the temporal expression of various molecules released from the polymer could be used to induce the differentiation of ES cells within the scaffolds (Richardson *et al.*, 2001). It has been shown that, by dual delivery of vascular endothelial growth factor (VEGF)-165 and platelet-derived growth factor (PDGF)-BB, each with distinct kinetics, from a single, structural polymer scaffold, a mature vascular network can be formed (Richardson *et al.*, 2001). An alternative approach to modify the surface that is exposed to the cells is to immobilize desired ligands on to the scaffold. For example, RGD peptides, the adherent domain of fibronectin, can be incorporated into polymers to provide anchorage for adherent cells (Cook *et al.*, 1997).

Another difficulty with the current materials is their lack of control over the spatial organization within the scaffold. To create tissues that resemble the natural structure of biological tissues, the spatial patterning of cells must be recapitulated. For ES cells differentiated in scaffolds, this modeling and structure may be directly obtained as cells differentiate. The spatial arrangement of cells grown in EBs is typically organized with cells of particular tissues appearing in clusters. For example, blood precursors occur in the form of blood islands, similar to their normal appearance in embryonic development. In the system in which ES cell-derived cells are plated onto scaffolds, spatial rearrangement can occur via direct patterning or cell "reorganization." In the direct cell patterning system, cells can be seeded into the scaffold at particular regions within the cells. For example, the direct attachment of two different cell types on different sides of the scaffold has been used to generate cells of the bladder (Lanza *et al.*, 2000). Cell patterning techniques, as have been developed for soft lithography for controlled co-culture of hepatocytes and fibroblasts, could be scaled-up to tissue-engineering scaffolds to allow for more controlled and complex direct patterning (Bhatia *et al.*, 1997, 1998a, b).

CONCLUSIONS AND FUTURE PERSPECTIVES

ES cells have generated a great deal of interest as a source of cells for tissue engineering. However, there are a number of challenges that exist in making ES cell-based therapy a reality. These include directing the differentiation of ES cells (using controlled microenvironments or genetic engineering), to ensure their safety and efficacy *in vivo*, to ensure that the cells are immunologically compatible with the patient and will not form tumors, to improve protocols for isolating desired cell types from heterogeneous populations, to enhance current tissue engineering methods. Further research is required to control and direct the differentiation of ES cells, in parallel with developing methods to generate tissues of various organs, and this may lead to realization of the ultimate goal of tissue engineering. We are getting close to a day when ES cells can be manipulated in culture to produce fully-differentiated cells that can be used to create and repair specific organs. Clearly, significant challenges remain, and the ability to overcome these difficulties does not lie within any scientific discipline, but rather involves an interdisciplinary approach. Innovative approaches to solve these challenges could lead to an improved quality of life for a variety of patients that could benefit from tissue engineering approaches.

KEY WORDS

Biomaterials Any natural or synthetic materials that interface with living tissue and/or biological fluids.

Cell therapy The transplantation of cells from various sources to replace or repair damaged tissue and/or cells.

Differentiation The developmental process in which cells change their genetic programs to become more restricted in their potential and to mature into functional cells of various tissues and organs.

Embryonic stem cells Cells derived from the embryo that can self-renew and give rise to all the cells of the adult organism.

Scaffold A porous structure typically made out of degradable polymers within which cells are seeded. Scaffolds provide geometrical structure for cells to reorganize and form 3D multicellular tissues.

Tissue engineering The interdisciplinary field that applies the principle of engineering and the life science toward the development of biological substitutes that restore, maintain, or improve tissue function.

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Chapter 64

Postnatal Stem Cells in Tissue Engineering

Pamela Gehron Robey and Paolo Bianco

The last decade has witnessed the emergence of stem cell-based tissue engineering as a means to restore normal structure and function to tissues lost due to trauma or disease. Successful translation to clinical application will rely on: (1) further characterization of the biological properties of post-natal stem cells (what they can and cannot do); (2) optimization of their isolation and *ex vivo* expansion; (3) formulation of appropriate scaffolds and carriers that may also include growth factors; (4) development of more advanced bioreactors to create tissues with appropriate biomechanical properties; and (5) determination of mechanisms by which to mobilize and activate endogenous stem cells. This review will highlight these aspects of stem cell-based tissue engineering, along with examples of current approaches and applications.

INTRODUCTION

In its first inception, tissue engineering was based on the use of natural or synthetic scaffolds seeded with organ-specific cells *ex vivo*. This approach was somewhat distinct from guided tissue regeneration, which utilized scaffolds and/or bioactive factors to encourage local cells to repair a defect *in situ*. These two approaches are now merged into the current field of tissue engineering, which encompasses multiple and diverse disciplines to use cells, materials, and bioactive factors, singly or in various combinations, to restore and even improve tissue structure and function. Stem cell-based tissue engineering represents a major turn in the conceptual approach to the reconstruction of tissues. Advances in stem cell biology are having a profound impact on tissue engineering at large through the extension of the repertoire of available cells, identification of potential applications, technological means of generating functional tissues *ex vivo*, and above all by making it possible (or promising) to engineer tissues that otherwise would not be amenable to recreation. In many cases, tissue engineering seems to have more use for stem cells than nature itself. This is especially apparent in the case of stem cells derived from tissues with low turnover, or no apparent turnover at all. If neural stem cells were able to repair the loss of dopaminergic neurons in the intact brain *in vivo*, there would be no Parkinson's disease for which to envision stem cell-based therapies. Dental pulp stem cells do not regenerate primary dentin *in vivo*, but vast numbers of cells can be made from the pulp of a single extracted tooth to generate copious amounts of new dentin. Current definitions of stem cells in fact include a technological dimension in many cases (a cell that can be expanded *ex vivo* and bent to generation of differentiating cells). Importantly, the two neighboring fields of cell therapy (reconstruction of functional tissues *in vivo* using cells) and tissue engineering proper (reconstruction of functional tissues using cells and something else) merge significantly once a stem cell angle is adopted for either, not only with respect to the ultimate goals, but also to several biotechnological aspects.

THE RESERVOIRS OF POSTNATAL STEM CELLS

Both for ethical constraints, and for ease of harvest and control of differentiation (which is not yet straightforward for human embryonic stem cells, or inducible pluripotent cells), current approaches to stem cell-based tissue engineering largely rely on the use of postnatal stem cells. Once restricted to a handful of constantly (and rapidly) self-renewing tissues, such as blood, skin, and the gastrointestinal track, the sources of postnatal stem cells have extended to include perhaps every single tissue in the body, regardless of the rate of tissue turnover, or ability to regenerate. Not all of these tissue-specific stem cells, however, are equally accessible for safe harvest, or available in sufficient quantity (or amenable for *ex vivo* expansion) to generate the number of cells needed for tissue regeneration. However, lessons on the dynamics of tissue homeostasis (growth and turnover) that can be learned from these cells

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have an obvious impact on the design of future tissue engineering strategies. In addition, mechanisms whereby postnatal cells maintain differentiated functions in tissues and organs are relevant to future embryonic stem cell-based approaches, and can only be learned from postnatal cells.

Plasticity and Transdifferentiation: Implications in Tissue Engineering

The clamor during the past few years concerning the potential "plasticity" (the ability of a cell to convert from one type to another) and "transdifferentiation" (conversion of a cell from one lineage to another) of certain classes of postnatal stem cells has suggested aditional possibilities for the use of postnatal stem cells in tissue engineering. However, rigorous proof of true plasticity and transdifferentiation have been lacking in many reports, and can only be demonstrated by the ability of a single (clonogenic) cell to form cells of multiple different phenotypes, and that they can be shown to function as those different cell types. Some would also add that conversion must be more than an isolated phenomenon, occur at a high frequency, and be persistent or stable. Even if postnatal stem cell plasticity or transdifferentiation could be rigorously demonstrated, other perspectives for usage in tissue reconstruction would need to be directly addressed. It would be impractical to say the least, to use neural stem cells for bone marrow transplantation, and thus, a tissue engineer's enthusiasm for this unexpected finding may remain lukewarm. In contrast, deciding whether liver regeneration is successfully accomplished using previously unknown "hepatogenic" stem cells, by transdifferentiation of hematopoietic stem cells, or by reprogramming of a donor lymphocyte nucleus following cell fusion, may not disrupt a tissue engineer's sleep, as long as the goal is met. Unquestionably, some examples of "unorthodox" differentiation of postnatal stem cells have been reported. Current evidence suggests that postnatal stem cells display limited plasticity, and only very rarely "transdifferentiate." However, even if this is true, transdifferentiation is exceedingly rare; analyzing the way that it occurs may provide clues as to how to manipulate cell populations better to increase its frequency.

Bone Marrow: A Source of Multiple Stem Cells

It has long been known that bone marrow is the home of at least two different types of stem cells, the hematopoietic stem cell (HSC), and the skeletal stem cell (SSC, also known as the bone marrow stromal stem cell, or "mesenchymal" stem cell), recently identified as CD146+ pericytes; each are able to reconstitute the hematopoietic and skeletal system, respectively. Both of these types of postnatal stem cells were thought to be able to contribute differentiated cell types outside of their physiological progeny. Highly-purified HSCs were reported to give rise to cardiomyocytes, as well as hepatocytes, and a host of epithelial tissues. Bone marrow stromal cells have been reported to generate functional cardiomyocytes *in vitro* (murine), and to be capable of neural differentiation. A rare subset of murine bone marrow cells (multipotential adult progenitor cells, MAPCs) have been reported to almost be as multipotent as ES cells, although it is not clear that these cells exist *in vivo*, or whether they are created by certain culture conditions *in vitro*. A subset of hematopoietic cells expressing CD133 has been found to differentiate into endothelial cells, and may represent a postnatal form of a hemangioblast. Marrow-derived cells infused systemically contribute to regeneration of skeletal muscle in response to injury, and in mouse models of muscular dystrophy. Donor-derived cells have also been detected in neuronal tissue, newly-formed vasculature, in the kidney, and even in the oral cavity following bone marrow or mobilized peripheral blood transplantation. Ideally, the identification of a single accessible site that would contain reservoirs of cells with pluri- and multipotentiality that are easily harvested in large quantities would mark a major advance in tissue engineering. These initial observations make bone marrow the central organ of tissue engineering and stem cell therapy (Figure 64-1). However, much more investigation is needed to determine the true

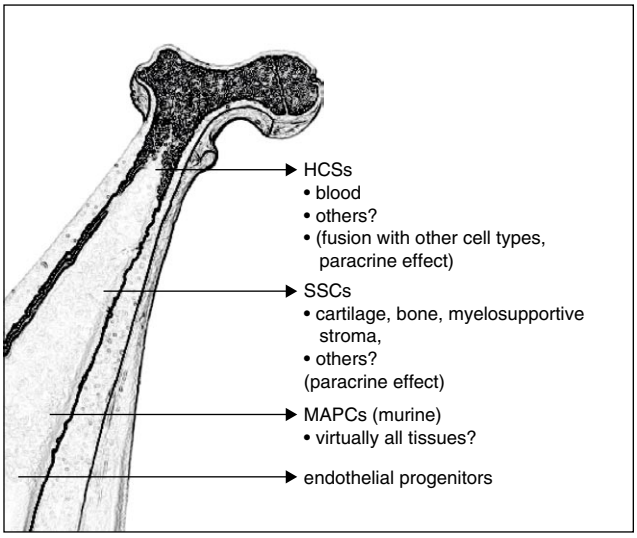


FIGURE 64-1 Bone marrow as a central source of postnatal stem cells. Bone marrow consists of at least two well-defined populations of postnatal stem cells, the hematopoietic stem cell (HSC) and the skeletal stem cell (SSC), both of which form numerous phenotypes within their cellular system, but may also rarely form cells outside of them. These HSCs and SSCs also have paracrine affects on local stem/progenitor cells and encourage repair processes. Multipotential Adult Progenitor Cells (MAPCs) are a subset of bone marrow stromal cells, and have been reported to form virtually all cell types in mouse. Endothelial precursors (CD133+) have been identified. These findings place bone marrow high on the list of tissues that are easily accessible in sufficient quantity for use in tissue engineering.

efficacy of bone marrow for treatment of such a broad spectrum of abnormalities. Based on more recent studies, it appears that exogenous bone marrow-derived cells may have a moderate benefit in treatment of diseases, but due to a "trophic" effect on local stem/progenitor cells, rather than due to transdifferentiation.

CURRENT APPROACHES TO TISSUE ENGINEERING

Tissue engineering approaches to the regeneration of functional tissue using postnatal stem cells can be envisioned by three different scenarios: (1) expansion of a population *ex vivo* prior to transplantation into the host; (2) recreation of a tissue or organ *ex vivo* for transplantation; and (3) design of substances and/or devices for *in vivo* activation of stem cells, either local or distant, to induce appropriate tissue repair (Figure 64-2). In all of these cases, considerable knowledge of the stem cell population's dynamics is required, in order to predict and control their activity under a variety of different circumstances.

Ex Vivo Culture of Postnatal Stem Cells

Ex vivo expansion of tissue- or organ-specific cells, used either alone, or added to soluble carriers or three-dimensional scaffoldings, or with growth factors, at the time of transplantation has been the primary approach in tissue engineering to date. However, *ex vivo* expansion in a fashion that maintains an appropriate proportion of stem cells within the population is a significant hurdle that must be overcome. For example, in spite of enormous efforts, the culture conditions for maintaining HSCs (let alone expanding their number) are as yet undefined. It is perhaps for this very reason that there are currently only a handful of examples in which *ex vivo* expanded postnatal stem cells are used successfully to restore structure and function. The key to successful expansion will lie in understanding cell proliferation kinetics (asymmetric versus symmetric division). The efficacy achieved by the use of *ex vivo*-expanded populations, whether stem or more committed in character, may also depend, at least in part, on the nature of the tissue under reconstruction. Within this context, the rate of tissue turnover most likely defines the rate of success. In tissues with a high rate of turnover, such as blood and skin, it is

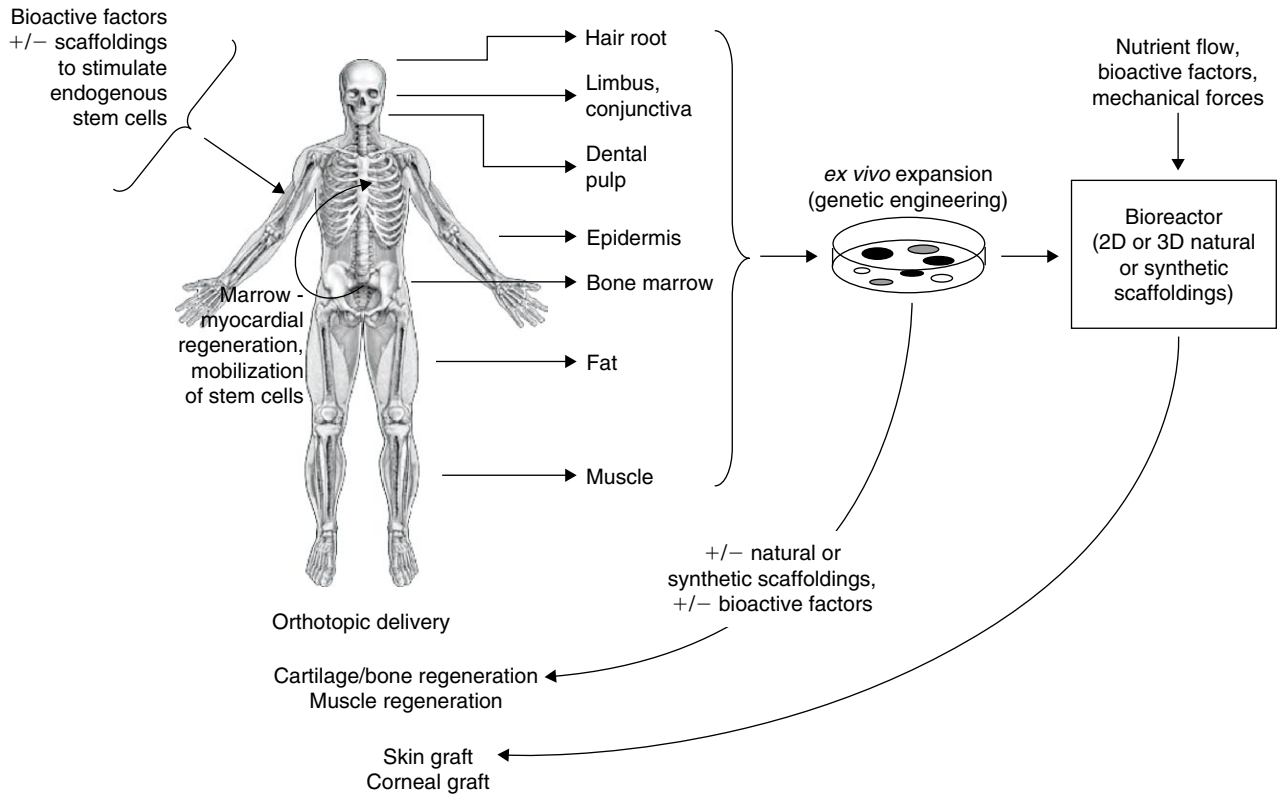


FIGURE 64-2 Current applications of postnatal stem cells in tissue engineering. While virtually all tissues in the body have cells with some regenerative capabilities, current postnatal stem cell-based strategies, or ones in the foreseeable future, rely on a relatively limited number of source tissues. Autologous bone marrow is currently in trial for myocardial regeneration. Most approaches utilize *ex vivo* expanded cell populations that are then delivered orthotopically in various combinations with bioactive factors and scaffolds, with skeletal and muscle regeneration. Generation of two-dimensional and three-dimensional structures *ex vivo* requires the use of bioreactors, in which cells are seeded onto scaffoldings and subjected to nutrient flow, bioactive factors and mechanical forces to induce formation of functional tissue for transplantation.

clear that long-term success is absolutely dependent on the persistence of a stem cell within the transplanted population. More committed progenitors may provide some short-term advantage, but without a self-renewing population failure is guaranteed. However, in tissues that turnover more slowly (e.g., bone), long-lasting benefit may be achieved with more committed populations of cells.

While optimizing culture conditions represents one hurdle, the issues of time and quantity represent others. In most cases, the amount of time required to generate the number of cells sufficient to repair defects induced by trauma or disease is in the order of weeks. In cases of trauma, this poses a large problem if the use of autologous stem cells is considered. For that reason, the use of allogeneic populations that could be used "off-the-shelf" from unrelated donors would be preferable, but this raises the issue of rejection, as in organ transplantation. Although it is suggested that some postnatal stem cells, in particular bone marrow stromal ("mesenchymal") stem cells, appear to escape from immune surveillance in allogeneic settings and are immunomodulatory, it is apparent that even autologous cells are lost when transplanted into sites other than from the stem cell's origin. In addition, differentiation of stem cells would imply the expression of a mature tissue-specific phenotype, including a complete histocompatibility profile. Use of allogeneic cells would most likely require concomitant immunosuppressive therapy, which has its own list of side-effects. However, cotransplantation of allogeneic bone marrow to reconstitute the hematopoietic system has been proposed as a way of inducing tolerance. Recent studies in organ transplantation, in conjunction with bone marrow, or mobilized blood transplantation after immune ablation, indicate a substantial improvement in long-term survival. In this type of approach, allogeneic cells could be better envisioned, provided that a single donor could be the source of cells for the tissue to be restored, along with the immune system.

Delivery of Stem Cells

Delivery of bone marrow or mobilized blood by systemic infusion for restoration of the hematopoietic system by HSCs is clearly an example of the efficacy of this method of delivery, but it is perhaps the only example. The success of systemic infusion is based primarily on the fact that, in terms of physical structure, blood is a simple, fluid entity. Bone marrow transplantation has been attempted for the delivery of nonhematopoietic cells to sites of injury, but it is not yet clear that there is long-term survival and engraftment of such cells. Furthermore, reconstruction of two- or three-dimensional structures requires different approaches. Currently, there are a number of clinical applications, or ones that are soon to emerge, for the delivery of cell populations orthotopically into a site for tissue regeneration. In some cases, specific populations of cells are expanded *ex vivo*, and delivered either alone, or in conjunction with a natural or synthetic material. As important as it is to control the *ex vivo* behavior of the cell population, it is also important to take into consideration what their response will be to the host environment. It is the host that must provide the signals that will dictate the differentiation, if uncommitted populations and a non-instructive carrier or scaffold are utilized. Furthermore, the recipient host tissue must also support the maintenance of the stem cell's niche, if not the creation of it *de novo*.

Reconstruction of the Skeletal Tissues: Bone, Cartilage, and Teeth

After numerous preclinical studies in a variety of animal models, it was demonstrated that bone marrow stromal cells, when used in conjunction with appropriate carriers, could regenerate new bone in critical sized defects that would never heal without intervention. A wide variety of carriers were tested, ranging from collagen sponges, synthetic biodegradable polymers to synthetic hydroxyapatite derivatives, although to date, none appear to be optimal. A carrier that provides immediate stability, especially in the case of weight-bearing bones, and yet can be completely resorbed as new bone is formed and remodeled, has not yet been fabricated. Nonetheless, a small number of patients were treated with *ex vivo* expanded BMSCs, in conjunction with hydroxyapatite/tricalcium phosphate ceramic particles, with good outcome, and other clinical studies are in progress (see ClinicalTrials.gov). In addition to healing segmental defects, bone marrow stromal cells (BMSCs) were also used to construct vascularized bone flaps. In cases where morbidity of the recipient site is an issue, it can be envisioned that a new bone rudiment could be grown elsewhere in the body and then transferred, with vasculature intact. More recently, adipose tissue was identified as a source of stem cells that also have the potential to differentiate into bone and cartilage at least, and that may be equally as multi-potential as BMSCs. Naturally, if these studies are further substantiated, fat would become another prime source of easily accessible stem cells for tissue engineering.

Current cell-based therapy for the treatment of cartilage defects relies on the growth of chondrocytes from a biopsy, followed by transplantation onto articular surfaces in conjunction with a periosteal flap. While this procedure is practiced worldwide, long-term efficacy is questionable, and there is a clear need for better procedures. BMSCs and stem cells from adipose tissue are capable of forming cartilage in a test tube, but their use in reconstruction of cartilage on articular surfaces will rely on inhibiting their eventual differentiation into hypertrophic chondrocytes. Further development of suitable carriers that provide the appropriate microenvironment to maintain the cartilage phenotype and prevent hypertrophy will be essential. Natural polymeric gels, such as hyaluronic acid, collagen,

alginate, and chitosan, provide an adequate three-dimensional structure to maintain the chondrocyte phenotype, but are not well modeled into specific shapes, and have very poor biomechanical properties. For these reasons, synthetic biodegradable polymers, such as polylactic acid and polyglycolic acid, and mixtures of both that can be crosslinked and molded to form porous three-dimensional structures, are thought to provide more adequate scaffolding, and have been used to construct cartilage with predefined shapes. In designing constructs of stem cells and scaffolds, the differences between the elastic cartilages of the ear and nose and articular cartilage must also be considered, to ensure that tissues form with the appropriate biomechanical characteristics.

Using techniques developed for isolation and characterization of bone marrow stromal cells, dental pulp from the permanent dentition was found to contain a population of stem cells (dental pulp stem cells, DPSCs) that have the ability to form copious amounts of primary dentin and a pulp-like complex upon *in vivo* transplantation with hydroxyapatite/tricalcium phosphate ceramic particles. Stem cells are also present in the pulp of deciduous teeth (SHED, Stem cells from Human, Exfoliated, Deciduous teeth) that have a high proliferative capacity, and in addition to forming dentin, may be able to form other cell types such as bone, or neural cells, perhaps due to the neuroectodermal origin of dental pulp. Cells isolated from the periodontal ligament recreate a PDL-like structure, and form cementum when transplanted in conjunction with hydroxyapatite/tricalcium phosphate, and recent studies have shown their utility in re-establishing periodontal tissues in animal models when transplanted in conjunction with stem cells isolated from the apical papilla (SCAPs). Based on these findings, reconstruction of structures in the craniofacial complex can be envisioned.

Regeneration of Skeletal and Cardiac Muscle

Muscle contains a population of muscle-specific stem cells, the satellite cells. Early attempts to use *ex vivo*-expanded populations of allogeneic myoblastic cells derived from satellite cells to correct muscular dystrophies were all uniformly unsuccessful, due to immunological responses of the host to allogeneic cells (and the expression of a foreign protein by the donor cells), and the inability of donor cells to repopulate extensively. Furthermore, satellite cells have limited ability to populate large areas on direct injection, and are unable to escape from the circulation on systemic injection, factors which limit their therapeutic application to date. In other studies, bone marrow transplantation provided a cell that could participate in the formation of new myotubes in a muscle injury model, and in a model of muscular dystrophy, however, the level of incorporation of donor cells was very low. A number of different cell types, including "mesenchymal" stem cells, have been found to fuse with myoblasts *in vitro*, and with myotubes *in vivo* (e.g., MSCs from synovial membrane), however this appears to be due to the natural process of cell fusion during muscle repair, rather than due true myogenic capacity.

Although skeletal muscle cells are functionally quite distinct from cardiomyocytes, autologous myoblastic cells have been viewed as a potential therapy for the treatment of myocardial infarct. While initial studies were encouraging, more recent clinical trials have failed to demonstrate improvement in global and regional cardiac function, due to a low rate of cell retention, high levels of cell death, and the inability of the engrafted cells to electromechanically link with recipient cells. It is also speculated that only a subset of myoblasts may be functionally useful. The utility of autologous bone marrow for myocardial regeneration is also currently receiving a great deal of attention, based on the purported ability of HSCs and BMSCs to form cardiomyocytes, and the ability of marrow-derived endothelial precursors to participate in the development of new blood vessels in a number of animal models. Based on these encouraging findings, treatment of ischemic heart failure using marrow-derived cells (either whole bone marrow or isolated subpopulations thereof) is being tested in a number of clinical trials around the world. For example, patients receiving an autologous bone marrow transplant in conjunction with cardiac bypass surgery, and those receiving CD133+ endothelial precursor cells alone, are reported to have increased cardiac perfusion and enhanced ventricular function. However, while the results indicate that use of bone marrow-derived cells is safe, it has been difficult to assess the level of efficacy, due to differences between studies in the clinical status of the patients, the sub-populations of cells utilized, the route of cell administration, and the outcomes by which to determine efficacy. Current evidence indicates that bone marrow-derived cells do not undergo substantial transdifferentiation into cardiomyocytes, or persist for long periods of time after injection or infusion, but that the benefit that they provide is based on the secretion of cytokines and growth factors that encourage the growth of endogenous stem/progenitor cells to initiate repair, a paracrine effect. Considering the high prevalence of ischemic heart disease, further refinement of the types of cells within bone marrow, used singly or in combination, along with the timing and route of delivery are ongoing. In addition, studies aimed at optimizing the paracrine effect through molecular engineering are also underway (e.g., over-expression of the survival factor, Akt, in bone marrow stromal cells).

Ex Vivo Reconstructions: Cells, Scaffolds, and Bioreactors

The creation of tissues, and even organs, prior to transplantation is a rapidly expanding area of research. This

approach relies on expanding cell populations on, or in, either natural or synthetic scaffolds in a bioreactor. Whether natural or synthetic, optimal scaffolds must be biocompatable, bioresorbable and nonimmunogenic. Furthermore, they must also be instructive, and dictate appropriate cell growth and differentiation, and support the recapitulation of a stem cell niche, which is essential for tissue renewal on transplantation. The most commonly used natural scaffolds include either individual purified extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin (or peptides derived from them), or devitalized ECM from skin, submucosa of the small intestine, urinary bladder, and others, and can be autologous, allogeneic, or even xenogeneic. Such devitalized ECMs contain not only the structural proteins (which also have other important biological activities) that define the three-dimensional organization of the tissue, but also the local repertoire of growth factors that are stored within them. Various chemical and nonchemical treatments have been applied to ECMs and their components in attempts to modify their biomechanical and immunological properties on transplantation, but these have resulted in less than desirable effects, due to inactivation of their conducive and inductive properties, and inhibition of their resorption and replacement. If left unmodified, ECMs generally promote infiltration and proliferation of cells and differentiation *in vitro* and *in vivo*, and are ultimately turned over on transplantation. Synthetic scaffolds are designed not only to mimic the biological properties of ECMs, but also to have enhanced material properties appropriate for a particular tissue. Polyglycolic acids, polyhyroxyalkonates, and hydrogels are the most common examples, and all can be manipulated to form a broad range of structures with varying degrees of porosity and rigidity. In addition, recent advances in nanotechnology have led to a variety of approaches for the development of engineered ECM analogs. Self-assembly, phase separation, and electrospinning of biomaterials are being utilized for the fabrication of nanofibrous scaffolds that recreate a natural microenvironment. Furthermore, scaffolds (natural or synthetic) can also be formulated to include morphogenetic and growth factors, or even naked plasmid DNA to transduce in-growing cells to produce these important factors, such that they can become instructive and biomimetic.

In addition to having appropriate scaffolds, the ability to construct tissues and organs *ex vivo* also depends on the design of appropriate bioreactors. The initial designs primarily provided nutrient flow through the developing tissue. However, many tissues grown under these circumstances failed to achieve the biomechanical properties required for tissue function *in vivo*. For example, the construction of blood vessels using a variety of biomaterials, and cell populations using culture perfusion, yielded structures that resembled native blood vessels histologically. Yet, when transplanted, these constructs failed, due to their inability to withstand changes in pressure. A substantial improvement in such constructs was achieved by subjecting the developing tissues to pulsatile flow conditions. Perfusion-type bioreactors support the growth of tissue with only a nominal number of cell layers, and are amenable for construction of relatively simple structures. Construction of larger, more complex, three-dimensional structures requires the development of even better bioreactors that will support cell survival to achieve a significant cell mass. Another critical aspect of using large constructs is the development of supporting vasculature. While constructs can be placed in vascular beds in some cases, it will be essential to design constructs that rapidly induce vascularization, perhaps by including the angiogenic factor, VEGF, or cells that produce it, or even by including endothelial cells and their precursors, which tend to assemble themselves into primitive tubular structures that may allow for more rapid establishment of organ perfusion.

Next to bone marrow transplantation, skin grafts perhaps provide the best example of the impact of postnatal stem cell biology on the success of cell-based tissue reconstruction. Although the use of *ex vivo* expanded keratinocytes for the generation of skin grafts was first brought to a clinical setting by the pioneering work of Green and co-workers, persistent engraftment was not routine. It is now recognized that both the expansion culture conditions in which the cells are grown, and also the scaffolding on which they are placed, must support the self-renewal of epidermal stem cells (cells that give rise to holoclones, as opposed to transiently amplifying meroclones, or more differentiated paraclones). Expansion of keratinocyte populations on fibrin substrates was found to maintain the stem cell population, and to improve long-term maintenance of skin grafts. There are now a number of studies underway to molecularly engineer autologous epidermal stem cells to correct a gene defect causing severe blistering prior to generation of skin grafts. Currently, there are a number of commercially-available products, some of which incorporate dermal fibroblasts in a collagen gel, which is then layered with *ex vivo* expanded epidermal cells. They provide coverage for severe wounds, and are maintained while the recipient generates new skin. The standard starting material for establishing epidermal cultures is skin; however, recent evidence indicates that a stem cell located in the bulge region of the root sheet of hair follicles has the ability to form not only new follicles and sebaceous glands, but also interfollicular epidermis under certain circumstances. These cells, which can be obtained non-invasively, are highly proliferative, and have been used successfully to treat leg ulcers. Based on lessons learned from skin regeneration, stem cells in the limbus of the sclera and conjunctiva have been expanded in culture on fibrin substrates to generate sheets of epithelial cells that are able to reconstitute a damaged corneal surface with dramatic beneficial clinical results.

Activation of Local and Distant Endogenous Stem Cells

While the *ex vivo* reconstruction of entire organs that are functional is the goal of the bioengineering world, what perhaps is even more challenging is the goal of inducing endogenous stem cells to become activated to reconstruct a tissue. Most tissues in the body display at least some sort of regenerative capability, but in many cases the capacity remains insufficient to mount a spontaneous and successful repair response *in vivo*. The application or induction of growth factors and morphogens, perhaps in combination with appropriate soluble carriers or scaffolds, might be envisoned to "encourage" local or distant progenitors to regenerate a functional tissue. This *in situ* process could occur through several different pathways, including activation of local or distant stem cells, or reprogramming of local cells, to generate adequate numbers of committed precursors. In all cases, a regulated morphogenetic process is needed to establish normal structure and function. Definition of the intrinsic properties of regenerative cells and extrinsic signals that may trigger a recapitulation of developmental processes is critical for the success of this approach.

Local Cells

Based on reasoning that, following an injury, signals that normally activate a local cell to initiate repair would be either obliterated or not of a high enough magnitude, numerous studies attempted to provide appropriate morphogenetic and growth factors. However, this approach has been uniformly disappointing, most likely due to the short half-life of such soluble factors and the lack of an appropriate carrier or scaffolding. Scaffolding provides not only a template for the organized outgrowth of local cells, but also a substrate on which to stabilize and/or orient factors (or parts thereof) for appropriate presentation to a cell. However, even if using "smart" constructs, tissue regeneration of large defects may not be complete, due to exhaustion of the local stem cell population, hence the need to maintain a stem cell niche. Furthermore, some clues as to the negative impact of local constraints on the native, albeit incomplete, repair capacity of cells and tissues may be found in studies of spinal cord regeneration. Following injury, there is a phase characterized by neurite outgrowth from the severed end; however, it is short-lived due to the production of inhibitory factors by cells within the myelin sheath, and gliosis impedes neurite outgrowth. Thus, regeneration by endogenous cells must not only take into consideration bioactive factors and scaffolding that are necessary to maintain the in-growing population with the appropriate balance of stem cells, transiently amplifying cells and differentiating cells, but also the inactivation of local inhibitory factors that work against the process.

Mobilization and Recruitment of Distant Stem Cells

Many studies (too numerous to cite here) have suggested that once introduced into the circulation, many types of cells have the propensity to home to a diseased or injured site, and participate in local regeneration, and it has been argued that HSCs and MSCs have the ability to form many cell types after systemic infusion, although not by rigorous criteria. Independent of these issues, a major question remains as to whether nonhematopoietic, extravascular cells can be induced to escape into the circulation due to trauma or disease. Likewise, it is not known what circumstances cause these cells to circulate, their origin and biological identity, the signals that mobilize them, and how they facilitate tissue regeneration.

Cytokine administration has become a well-established procedure for mobilization of HSCs into peripheral blood, but is not clear whether other stem/progenitor cell populations present in marrow (endothelial precursors, bone marrow stromal cells) would be equally amenable to liberation by current procedures, or whether different ones would have to be developed. It does appear that endothelial precursor cells, which are resident in bone marrow, can be mobilized by various growth factors and cytokines, and participate in vascularization in areas of trauma and disease. On the other hand, there is no rigorous evidence that bone marrow stromal cells escape into the circulation, although cells with BMSC-like character can occasionally be found in human peripheral blood, and in other animal species. It is not clear what relationship these cells have, in comparison to cells that are isolated based on cell sorting for osteocalcin, a secreted protein. Mobilization of nonhematopoietic marrow-derived stem cells may provide a mechanism to enhance a local population, and improve tissue regeneration. However, there is much to be learned in order to optimize their liberation into the circulation within the context of the nature of the injury or disease, and the timing in which to best introduce them.

CONCLUSIONS

The recent advances in embryonic and postnatal stem cell biology have most certainly captured the public's attention, and have raised expectations for miraculous cures in the near future. While certain applications that utilize postnatal stem cells are in practice or will be shortly, the majority are still in their infancy, and will take much more effort to bring to fruition. However, the current sense of urgency in translating recent findings into clinical applications should not lead this field to repeat the mistakes experienced in the field of gene therapy. The development of tissue engineering as a viable medical practice must proceed in an evidence-based fashion in all of the associated disciplines that are involved, and several hurdles are yet to be overcome.

First, our current understanding of postnatal stem cell biology is rudimentary, at best. The manner in which we isolate stem cells, and manipulate them and their progeny appropriately, relies heavily on a clear understanding of cell population kinetics. This also requires a complete understanding of their response not only to bioactive factors, scaffoldings and delivery systems, but also to the host microenvironment in which they must survive and function. Second, more efficient bioreactors that adequately model the microenvironment, and also allow for scale-up of the tissue engineering process, must be developed. Third, in order to translate what we learn into clinical application, development of appropriate preclinical models to prove the principle that stem cells do indeed have a positive biological impact is absolutely essential. In analyzing these models, stringent criteria must be defined to determine efficacy, and we must remain principled in assessing them. Exciting times, yes, but also a time for due diligence to bring what started off as a scientific curiosity into medical reality.

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KEY WORDS

Biomimetic Human-made processes, substances, devices, or systems that imitate nature.

Bioreactor An apparatus designed for the *ex vivo* construction of tissue or organ-like structures that provides appropriate three-dimensional orientation and nutrition to cells that are seeded within it. Current designs may also include the application of appropriate physiological stresses to "condition" the construct for better performance on *in vivo* transplantation.

Scaffold A temporary or permanent framework used to support cell growth and differentiation that may be natural or synthetic in nature, and may also include morphogens and/or growth factors that influence the pattern of proliferation and differentiation (biomateria).

Tissue engineering A field that encompasses multiple and diverse disciplines to use cells, materials and bioactive factors in various combinations, to restore and even improve tissue structure and function.

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Chapter 65

Stem Cell Gene Therapy

Brian R. Davis and Nicole L. Prokopishyn

In this chapter, we focus specifically on *ex vivo* gene therapeutic modification of autologous stem cells from patients with genetic disease and discuss the elements crucial for successful clinical application of these therapies. In particular, we contrast gene addition and genome editing approaches for stem cell gene therapy, highlighting the particular challenges that each approach faces to achieve therapeutic benefit.

INTRODUCTION

Primitive stem cells capable of self-renewing proliferation and single or multiple cell lineage progeny generation have been identified in several human and mouse tissues. For example, various stem cells individually capable of producing hematopoietic, mesenchymal, endothelial, or liver cells have been identified in adult bone marrow. Although the biological characterization of various nonhematopoietic stem cells is still in its early stages in the laboratory, therapeutic experience with hematopoietic stem cells (HSCs) suggests that other stem cell types will likely have successful clinical applications.

The HSC has exhibited the ability to establish a normal, healthy blood system in patients following transplantation of normal blood stem cells from closely-matched individuals—treating disorders of the blood system including immune deficiency, thalassemia, and leukemia. HSC gene therapy offers significant promise for treatment of various hematopoietic diseases, because genetic correction of autologous HSCs could result in long-term correction of blood system cells while avoiding the immune system complications resulting from nonidentical transplantation. For example, thalassemia and sickle cell anemia, the most common genetic diseases of blood, could potentially be treated, either by delivery of the globin transgene to HSCs, or by direct repair of a specific globin gene mutation in the HSCs.

Significant attention has been devoted to the isolation, culture, and genetic modification of human bone marrow-derived mesenchymal stem cells (MSCs), because they generate cells of cartilage, bone, adipose, marrow stroma, and possibly muscle. Provided that these genetically-modified cells, or their differentiated progeny, can be efficiently delivered to the required tissues *in vivo*, genetic modification of these cells is a potential treatment for various genetic diseases affecting mesenchymal cells, such as osteogenesis imperfecta, Marfan's syndrome, and muscular dystrophy. Identification of somatic stem cells giving rise to liver, pancreas, and brain raises the possibility of future applications of *ex vivo* stem cell gene therapy to treatment of diseases affecting these organ systems.

A recent significant development in the stem cell field has been the demonstrated ability to transform various human and mouse cell types—including adult skin cells—into pluripotent stem cells. Pluripotent cells have the ability to give rise to differentiated cells of all three germ layers: endoderm, ectoderm, and mesoderm. These induced pluripotent stem (iPS) cells exhibit most or all of the properties previously ascribed to embryonic stem cells, including the ability to proliferate extensively in tissue culture without loss of pluripotency, to generate various cell types present in different organ systems (e.g., cardiomyocytes, neurons), and in the case of mouse iPS cells, to develop into a complete mouse. Incredibly, this "reprogramming" of adult cells into iPS cells, first demonstrated by Dr Shinya Yamanaka of Kyoto, Japan, can be achieved by delivering only four transcription factors to the adult cells. This development has the ability to revolutionize the emerging field of regenerative medicine, by allowing the generation of patient-specific iPS cells from virtually any individual. These cells, once expanded in number and differentiated into the required cell type(s), could, in principle, be transplanted back into the same individual without the potential complications of transplant rejection. Significantly, for patients with genetic diseases, their iPS cells could be genetically-corrected in order to generate corrected, differentiated progeny cells for purposes of autologous transplantation.

Although one day it may be possible to target various stem cells specifically *in vivo* (e.g., based on a stem cell–specific surface phenotype), this possibility is not available today. Instead, it is more likely that the relevant autologous

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stem cells will first be isolated from tissues of affected individuals, and genetically modified *ex vivo*. The ability to isolate stem cells (hematopoietic and nonhematopoietic) from patients with genetic disease, genetically correct the stem cells, possibly expand them *ex vivo*, and transplant them back into patients, with the goal of producing genetically-corrected cells *in vivo*, offers significant potential for the genetic treatment of human disease.

Genetic modification in stem cells will be designed either to completely correct the genetic defect, or at least to compensate for the genetic defect. Gene defects occur in a variety of forms, ranging from a simple base pair mutation to the complete absence of a gene. Two general approaches can be used to correct defective genes within cells: gene addition and genome editing (outlined in Figure 65-1).

GENE ADDITION

Gene addition involves the delivery of corrective DNA (usually composed of the entire coding region of a gene and appropriate regulatory sequences) that compensates for or overrides the defective gene (Figure 65-1). The defective gene, unless it is completely absent, remains in the affected cells. This approach is particularly applicable when the endogenous gene product is not expressed (e.g., because of extensive deletion of the gene).

Successful application of stem cell gene therapy requires that therapeutic genes delivered to stem cells persist in the self-renewing stem cells, and in their mature and differentiated progeny. This requirement for transgene persistence is critical because: (1) transgenes present only transiently in stem cells undergoing self-renewing proliferation will only be of short-term therapeutic benefit; and (2) expression of the corrective gene likely exerts its effect in the differentiated progeny, which may be numerous cell divisions downstream of the genetically-modified stem cell. Transgene persistence can be accomplished, in principle, either by integration into one of the existing chromosomes, or by incorporation of the transgene in a synthetic human microchromosome.

Viral Vectors

Over the past two decades, significant attention has been devoted to the development of viral vectors and transduction protocols capable of stable introduction of genetic information into stem cells. Retrovirus, lentivirus, and adeno-associated virus (AAV) vectors are the most common vectors used in transduction of HSCs. After several years of disappointing results, recent reports in humans and other primates, most particularly the successful treatment of X-linked severe combined immune deficiency (SCID)

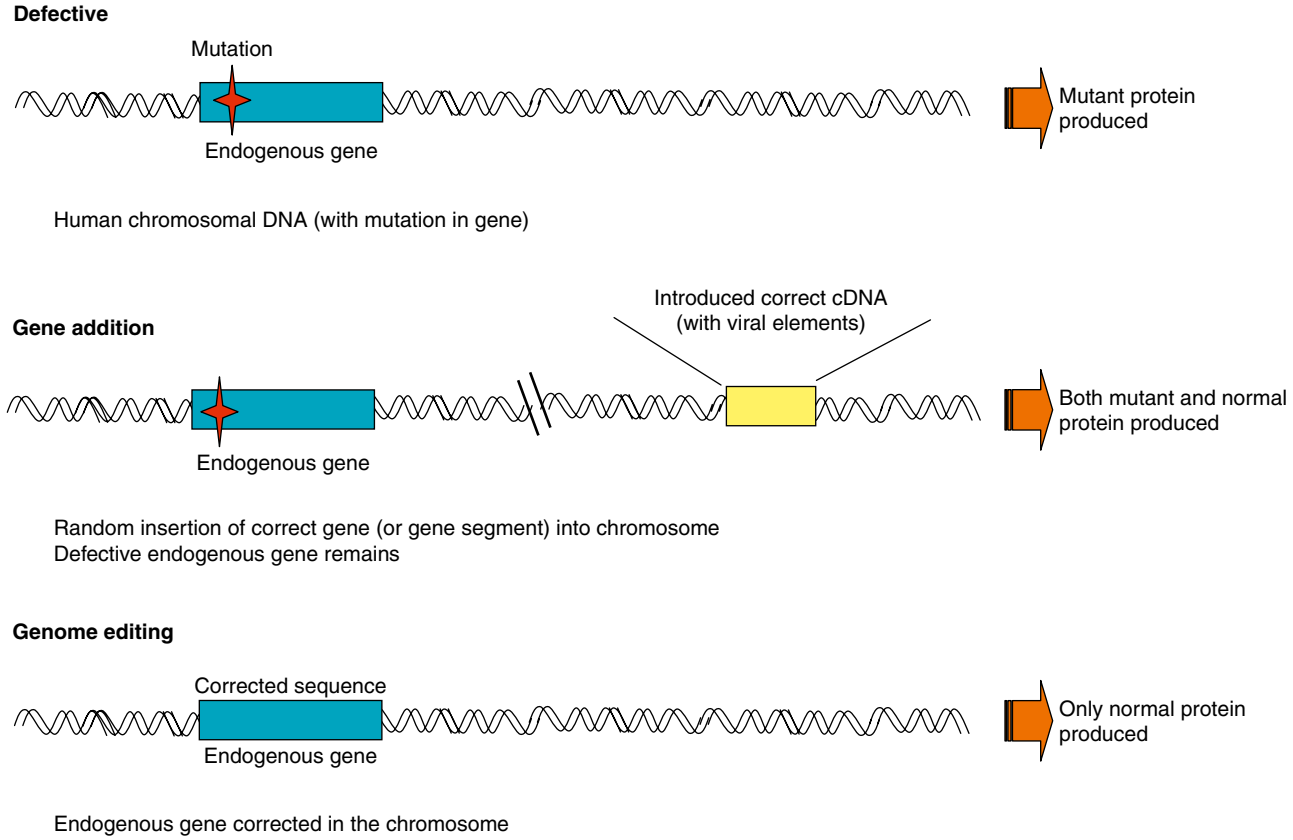


FIGURE 65-1 Schematic comparison of gene addition and genome editing approaches to stem cell gene therapy.

and adenosine deaminase deficiency (ADA), indicate that viral approaches can be successful in treating specific HSC-based diseases; however, a number of potential difficulties must be overcome to make this a safe and effective approach for stem cell gene therapy (discussed later).

Nonviral Integration Strategies

Other approaches have recently been developed that allow for integration into the genome without the use of viral vectors. Stable integration of large DNA sequences (including necessary regulatory elements) into specific "attP sites" in the genome can be accomplished using integrase from bacteriophage phiC31. This method may allow genetic correction of inherited diseases caused by mutations in large genes. Also, transposon-based systems (e.g., Sleeping Beauty) have been designed to allow for the insertion of transgenes into the human genome without the use of integrating viral vectors. However, optimal transposon size may prevent the inclusion of larger genes or necessary regulatory elements.

Synthetic Microchromosomes

Synthetic microchromosomes (SMCs), containing centromeric and telomeric sequences derived from functional human chromosomes, can accommodate insertion of genetic regions that are sufficient to house therapeutic transgenes, together with crucial intron/exon structure and regulatory sequences conferring appropriate transgene regulation. Whether transgene sequences maintained on SMCs are subject to the dysregulated expression and silencing that affect integrated sequences remains to be determined. Crucial issues with application of these SMCs to stem cell gene therapy include the efficient delivery of single-copy SMCs to target cells, and the persistence of these SMCs during cell division and differentiation.

GENOME EDITING

The second approach, genome editing, uses DNA repair and/or homologous recombination processes to correct an existing defective gene sequence, so that the defective or mutated area of the gene is restored to a corrected normal state. Genome editing (as shown in Figure 65-1) involves the delivery of DNA, modified DNA polymers, or linear single-stranded AAV vectors that are homologous to the target gene sequence with the exception of the base or bases intended for alteration. The genome editing process is directed by endogenous cellular machinery (potentially including mismatch repair and homologous recombination) acting at these target bases and the sequence mismatches created. The target bases are exchanged for the bases present on the introduced DNA—correcting or repairing the gene. The genetic alterations exacted are specific, targeted, and permanent. Repairing the defective sequence itself maintains the corrected genetic material within its normal chromatin environment, ensuring appropriate genetic regulation and expression in the cell. Genetic diseases resulting from well-defined, limited alterations in the DNA sequence, such as sickle cell disease, are ideal candidates for genome editing-based gene therapy strategies. Genome editing may also be the only suitable strategy in situations in which mutant gene product exercises a dominant negative influence over the normal gene product. For example, overexpression of normal collagen cannot surmount the harmful effects of mutant collagen chains produced in the disease osteogenesis imperfecta.

Addition of accessory molecules may also provide for increased genome editing rates. For example, up to 100-fold increases in gene targeting mediated by DNA or AAV vectors have been observed following the introduction of DNA double-strand breaks with sequence-specific nucleases. One demonstrated approach for generating novel sequence-specific nucleases has been to link a DNA-binding domain (itself composed of 3–4 zinc fingers, each of which bind specific DNA sequences), together with a DNA cleavage domain. Another approach has been to mutagenize the DNA binding domain of an existing endonuclease, to create other nucleases with altered DNA sequence specificity.

REQUIREMENTS FOR SUCCESSFUL STEM CELL GENE THERAPY

Genetic Modification Directly in Stem Cells

For long-term therapeutic benefit, it will typically be insufficient to modify only progenitor or differentiated cells; instead, direct genetic modification in stem cells will be required for sustained availability and production of corrected progeny cells. It is also essential that this genetic modification is accomplished without loss of stem cell activity.

Gene Addition

Since the mid-1980s, retrovirus vectors have been the vehicles of choice for delivering transgenes (typically cDNAs, together with limited transcriptional regulatory sequences) to cells, because they facilitate efficient transgene delivery and integration into the chromosomal DNA of proliferating target cells. The particular challenge of transducing quiescent HSCs was a primary impetus for significant improvements in transduction technology, optimization of *in vitro* transduction conditions including choice of cytokines and use of retronectin, use of various viral envelopes, and development of lentivirus and AAV vectors. These transduction methods allow for delivery without loss of stem cell activity. The limited packaging size of retroviral, lentiviral, and AAV vectors precludes packaging of certain

cDNAs (e.g., the complete dystrophin cDNA is too large to be incorporated into the previously-mentioned vectors). The packaging of cDNAs means that only one gene product will typically be expressed, as opposed to the possible expression of alternatively spliced forms from the normal genomic configuration.

Genome Editing

Stem cell genome editing requires delivery of genome editing molecules to stem cells without loss of stem cell function, and successful editing of genomic sequences directly in the stem cells. Ideally, one would want efficient, quantitative delivery of genomic editing molecules to the nuclei of stem cells. Typical nonviral macromolecule delivery methodologies face inherent and/or potential limitations in human stem cells. For example, electroporation or liposome-mediated transfection conditions have not yet been reported that allow for the efficient delivery of macro-molecules to HSCs without significant loss of viability or stem cell function. Thus, sequence-specific nucleases and donor DNA molecules will typically be delivered to stem cells with non-integrating viral vectors (e.g., adenovirus, integration-defective lentivirus, AAV).

It is not presently known whether the efficiency of editing chromosomal sequences is affected by the cycling status of stem cells. Although not an issue for those stem cells that can apparently be maintained in a proliferative state *in vitro* without loss of stem cell function (e.g., MSCs and iPS cells), the question of whether replication of the target gene chromosomal DNA is required for successful genome editing is important for quiescent HSCs. It is perhaps important to note that the various genome editing molecules summarized previously may use different mechanisms for genome editing and may, therefore, exhibit different requirements for cycling. It also remains to be determined whether efficient genome editing requires active transcription and/or an open chromatin conformation of the target gene. Although some genes requiring modification will already be expressed in stem cells (e.g., housekeeping genes), there are other target genes that would normally not be expressed until a certain state of differentiation or activation has been reached. Although it is assumed that stem cells in a quiescent state normally perform ongoing surveillance and repair of chromosomal mutations in both transcriptionally active and inactive genes, to our knowledge this has not been directly examined.

Genetically Corrected Stem Cells and Their Relevant Differentiated Progeny Consistently Present at Sufficient Frequency *In Vivo*

A critical issue in stem cell gene therapy is achieving and maintaining a level of corrected stem cells and/or their progeny *in vivo* sufficient to achieve the desired therapeutic effect. Transplanted genetically-modified stem cells and their progeny will exist *in vivo* in a background of transplanted unmodified cells and endogenous, unmodified stem cells and their progeny. The percentage of "corrected" cells required for therapeutic value will differ for various diseases. Factors that will influence the percentage of genetically modified cells *in vivo* are: (1) the percentage and number of genetically modified stem cells *ex vivo* before transplantation; (2) the ability to expand the stem cells *ex vivo*; (3) the ability to selectively expand or select for the genetically-modified stem cells *ex vivo* or *in vivo* (at the expense of the endogenous, defective stem cells); and (4) whether the genetically-modified cells (either stem cells or their differentiated progeny) have a proliferative, survival, or functional advantage *in vivo*.

If there was a capability for significant *ex vivo* expansion of stem cells, without loss of stem cell biological activity, the number of genetically-modified stem cells delivered to the patient could be significantly increased, potentially increasing the frequency of genetically-corrected versus endogenous defective stem cells *in vivo* following transplant. In addition, if there existed the ability to obtain, *ex vivo*, expanded populations of cells all derived from a single stem cell clone, cells for transplantation could be prepared that were precharacterized for their site of retroviral integration (to eliminate clones with problematic insertional mutagenesis sites, or to identify integrants likely to demonstrate appropriately regulated transgene expression), or for successful genome editing (i.e., only delivering appropriately corrected stem cells). Studies performed with human MSCs or iPS cells suggest that these classes of stem cells may be capable of significant *ex vivo* expansion.

Gene Addition

Inclusion of a selectable marker gene (e.g., chemotherapeutic drug resistance gene), in addition to the therapeutic transgene, in the same viral vector will, in principle, permit *ex vivo* or *in vivo* selection of genetically-modified stem cells and their progeny. Although studies have demonstrated excellent *in vivo* selection for the genetically-modified cell, a concern remains with regard to potential *in vivo* toxicity at the time of chemotherapeutic treatment or incidence of cancer in later years, because of the chemotherapeutic treatment(s).

Genome Editing

Genome editing will, most likely, first find application when there is a natural *in vivo* selective advantage for genetically-corrected stem cells and/or their progeny (e.g., Wiskott Aldrich syndrome [WAS], Fanconi's anemia, or X-SCID). There should be a strong selective advantage

in vivo for the corrected HSCs and their T-lymphocyte progeny in WAS, as evidenced by studies of X-chromosome inactivation patterns in female WAS carriers, and the selective reversion to a corrected WASP allele in a patient with WAS. These data strongly suggest that even the correction of a small number of HSCs with the capability of differentiating into gene-corrected T-cell progenitors and mature T-lymphocytes may lead to significant clinical benefit in WAS. This suggestion is supported by recent efforts to treat X-SCID via stem cell gene therapy. It is believed that strong *in vivo* selective pressure for T- and B-cells, expressing the gamma chain of the interleukin (IL)-2 receptor, contributed to the success of the X-SCID trials.

For those diseases in which the genome edited stem cells and/or their progeny do not have a selective advantage *in vivo*, successfully genome edited stem cells could subsequently be transduced with constructs having selectable genes. These selectable genes could be excised by systems such as Cre-Lox following *in vivo* selection.

Physiologically Appropriate Expression Levels of the Corrected Gene Product in the Relevant Cells

Correction of genetic deficiencies will typically require that the therapeutic gene be expressed at the appropriate level in the relevant cells for the life of the patient. Genetic treatment of some diseases will have less stringent requirements for either the level of required expression (e.g., chronic granulomatous disease), or the ability to tolerate indiscriminate expression (e.g., ADA, Gaucher disease). In contrast, successful treatment of other diseases (e.g., hemoglobinopathies, X-linked agammaglobulinemia) will likely require expression that is both of sufficient level and cell type-specific. For example, it is expected that clinical benefit in hemoglobinopathies will require that nonerythroid cells remain absent of globin expression, and that most erythrocytes will express the inserted gene at greater than 10–20% of normal globin levels for thalassemia and 20–40% of HbF levels for sickle cell disease. Finally, ectopic constitutive expression of therapeutic transgenes may be harmful in cases in which expression of the endogenous gene is tightly regulated (e.g., with respect to the cell's activation state).

Gene Addition

Significant problems were initially encountered in satisfying the requirement for long-term, cell type-specific transgene expression. The expression of retrovirus-transduced transgenes is frequently silenced, or exhibits significant variability in the level of expression from cell to cell, in the progeny of transduced cells. The difficulty in achieving appropriate expression (proper level, regulation, and cell type-specificity) is due to integration position effects, uneven distribution of the number of integrated gene copies per cell, and the inability to package sufficient regulatory sequences within the viral vector. As a consequence of this dysregulated expression, cells, although genetically-modified with corrective genes, may not display the corrected phenotype efficiently. Several recent studies have reported that multiple retroviral or lentiviral integration events per cell were required for adequate levels of transgene expression. The requirement for multiple integration events creates at least two problems: (1) increased likelihood of insertion mutagenesis (see later); and (2) a potential for inappropriately high expression of transgene in the cells (e.g., an overexpression of β-globin may manifest as α-thalassemia).

Interestingly, these same features of dysregulated transgene expression were also observed in the early transgenic mouse expression studies. Subsequent studies demonstrated that long-term, position-independent, copy number-dependent, cell type-specific expression required strong promoter/enhancer elements, sufficient genomic sequences to confer the appropriate chromatin configuration dominantly, and sufficient intron/exon structure and sequences for high-level expression. The strict packaging requirements for retrovirus, lentivirus, and A AV vectors may preclude inclusion of sufficient regulatory sequences and/or intron/exon structure for therapeutic applications requiring highly-regulated gene expression.

The complex and coordinate regulation of globin gene expression *in vivo* raises significant challenges for treating sickle cell anemia or β-thalassemia by addition of a normal or specially designed globin gene using retroviral, lentiviral, or A AV vectors. Much effort has thus been dedicated to identifying and including in viral vectors the regulatory sequences that are necessary and sufficient for appropriately-regulated transgene expression. Recently, the utilization of optimized vector constructs has yielded significant improvements in long-term, erythroid-specific globin expression in mouse models. Whether these very promising results will be confirmed in human clinical trials remains to be determined.

A significant challenge for achieving appropriately-regulated transgene expression via stem cell gene therapy is that the transgene, together with regulatory sequences, is delivered into a chromatin environment (specifically, stem cell) that may be significantly different from the chromatin environment in which the transgene expression is ultimately required (e.g., erythroid for globin expression). For example, a transgene may integrate into an active chromatin locus in stem cells that may subsequently become an inactive chromatin locus in the differentiated cell type(s). Furthermore, it is not known whether transcriptional regulatory sequences directly incorporated into stem cell chromatin will show the same loading of chromatin remodeling and transcription factors as when the transcriptional regulatory sequences are present in the chromatin from the stage of embryonic stem cell onward. In other words,

is chromatin structure (including histones, transcription factors, etc.) a consequence of sequential steps of factor addition and removal, originating in the fertilized egg or embryonal stem cell? Or can it be created *de novo* from factors present in the HSC?

Genome Editing

Repairing the mutation itself within the defective gene (e.g., β-globin gene) would maintain the correct genetic material within its normal chromatin environment, and in principle ensure appropriate genetic regulation and expression in the progeny differentiated cells (e.g., eryth-roid cells). This is one of the primary advantages of the genome editing approach for genes in which the transcrip-tional regulatory sequences are intact.

Absence of Interference from Endogenous Defective Gene Product on the Activity of the Corrected Gene Product

For those situations in which there is ongoing expression of the endogenous defective gene, a critical issue is whether this mutant gene product will interfere with the function of the introduced correct transgene. For example, it is possible that endogenous expression of a truncated or mutant protein could interfere with the functioning of an introduced normal protein in a dominant negative manner (e.g., collagen or WASP). Persistence of the mutant protein may be a particular problem in cases in which the gene product normally forms homodimers, heterodimers, or trimers.

Gene Addition

Addition of a normal gene into a stem cell means that the defective gene is still present within that cell and is capable of action. If ongoing expression of the endogenous defective gene is detrimental to the cell, it may be necessary either to express a specific transgene product that is designed specifically to counteract the activity of the mutant gene (e.g., expression of an anti-sickling gene), or to overexpress the normal transgene product at a level sufficient to dilute out the effect of the mutant gene product. These approaches may not be possible in all cases, because overexpression of the normal gene product may itself generate negative side-effects or unexpected results.

Genome Editing

Genome editing may be the only suitable genetic modification in situations in which the mutant gene product exercises a dominant negative influence over the normal gene product—because this approach would convert the endogenous gene to a normal form—ablating the detrimental mutant gene.

Absence of Adverse Effects

The minimization of side-effects or adverse effects caused by the treatment itself is of critical importance in any therapy. A partial listing of potential adverse effects from stem cell gene therapy would include: insertional mutagenesis from transgene integration; nonspecific gene editing introducing untoward mutations; and inadvertent carcinogenesis from the *in vivo* selection method for corrected cells. Although a complete absence of any adverse effects is clearly the goal of any genetic therapy, the potential risks of any treatment must be assessed in conjunction with the potential benefits. For some of the diseases potentially amenable to stem cell gene therapy, the current prognosis is very poor with no other therapy available. It should also be remembered that the introduction of other groundbreaking therapies (e.g., heart transplantation, marrow transplantation) resulted in numerous initial failures, including the death of patients. In addition, even today, therapies such as allogenic marrow transplantation for primary immunodeficiency disease are only 90% successful.

Gene Addition

A difficulty of the gene addition approach is the potential leukemogenicity resulting from random integration of the introduced viral vector into chromosomal DNA (e.g., either activating oncogenes or disabling tumor suppressor genes). This possibility has been reported in the otherwise successful X-SCID human trial in France.

Genome Editing

One of the crucial criteria for genome editing is the specificity of the editing event. A balance between specific and nonspecific genome editing may occur, and as such it is imperative to develop methods that favor specific actions. Also, use of methods that introduce double-strand DNA breaks (believed to increase the rates of repair) requires strict regulation to ensure that these breaks are limited and specific to the target sequence (e.g., through engineering of site-specific nucleases). Some of the molecules used in genome editing also have the potential for random integration within the genome and, therefore, possess the potential to disrupt genes and/or cause mutagenesis. Assessment of this risk or application of molecules that do not randomly integrate into the genome will be necessary for therapeutic application.

CONCLUSION

Gene addition and genome editing approaches, as applied to human stem cells, offer significant therapeutic potential. It is most likely that clinical benefit will initially be demonstrated in diseases in which there is a clear selective advantage (e.g., proliferative, functional, or survival) for

the corrected stem cells or their progeny, and/or in which the requirements for regulation of gene expression are less stringent. Significant improvements in both technologies will be required to bring about therapeutic benefit to a wider range of inherited genetic diseases.

KEY WORDS

***Ex vivo* stem cell gene therapy** The process of isolating stem cells (hematopoietic and nonhematopoietic) from patients with genetic disease, genetically correcting the stem cells, possibly expanding them *ex vivo*, and transplanting them back into patients with the goal of producing genetically corrected cells *in vivo*.

Gene addition Delivery of corrective DNA (usually composed of the entire coding region of a gene and appropriate regulatory sequences) to cells containing the defective gene.

Genome editing Specific, targeted, and permanent correction of an existing defective gene sequence so that the defective or mutated area of the gene is restored to a corrected normal state by delivery of molecules that are homologous to the target gene sequence, with the exception of the base or bases intended for alteration. The genome editing process is directed by endogenous cellular machinery (potentially including mismatch repair and homologous recombination) acting at these target bases and the sequence mismatches created.

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Part VI

Regulation and Ethics

Chapter 66

Ethical Considerations

Ronald M. Green

INTRODUCTION

Human embryonic stem (hES) cell research is ethically controversial, because it entails the destruction of human embryos. Here, I consider and discuss a series of key ethical questions associated with this research. These include whether it is morally permissible to destroy a human embryo, and whether we should clone human embryos for therapeutic cloning research. I conclude by stating my own position, that hES cell and therapeutic cloning research are both acceptable—and I offer some guidelines that have been developed by several authoritative bodies for the actual conduct of this research.

There is consensus in the scientific community that hES cell research holds great promise for developing new treatments for a variety of serious and currently untreatable disease conditions (National Research Council, 2001; Office of Science Policy, 2001). However, because hES cell research requires the manipulation and destruction of human embryos, it has also been a focus of ethical controversy and opposition. In the course of these debates, several challenging ethical questions have been raised. Scientists, clinicians, or patients involved in hES cell research or therapies must formulate their answers to these questions. Society, too, must address them to determine the extent to which hES cell research may require oversight and regulation. Here, I present these questions, and examine some of the leading answers that have been proposed to them.

IS IT MORALLY PERMISSIBLE TO DESTROY A HUMAN EMBRYO?

Human embryonic stem cell lines are currently made by chemically and physically disaggregating an early, blastocyst-stage embryo, and removing its inner cell mass. At this stage, the embryo is composed of approximately 200 cells, including an outer layer of differentiated placental material, and the undifferentiated (pluripotent) cells of the inner cell mass. The embryo dies as a result of this procedure. Hence the question: can we intentionally kill a developing human being at this stage to expand scientific knowledge, and potentially provide medical benefits?

It is true that a method involving single-cell blastomere biopsy has been developed that could obviate the need to destroy the embryo in order to develop an hES cell line (Klimanskaya *et al.*, 2006). New technologies for producing induced pluripotent stem (iPS) cells (see below) hold out the promise of replacing the use of embryos altogether. Nevertheless, many existing hES cell lines were created by destroying embryos. There are also hundreds of thousands of frozen embryos that could still be used to create new lines (Weiss, 2003). These realities make the question of the embryo's moral status, and the ethics of its destruction, still relevant.

At one end of the spectrum are those who believe that, in moral terms, human life begins at conception. For those holding this view, the early embryo is morally no different from a child or an adult human being. It cannot be used in research that is not to its benefit, and it cannot be used without its consent (The Linacre Centre for Healthcare Ethics, 2001; Pope John Paul II, 2003). Furthermore, proxy consent by parents in such cases is inadmissible, since it is an accepted rule of pediatric research that parents may not volunteer a child for studies that are not potentially beneficial, and that risk the child's life. Many Roman Catholics, evangelical Protestants, and some Orthodox Jews take the position that life (morally) begins at conception, and they oppose hES cell research. At the other end of the spectrum are those who believe that the embryo is not yet fully a human being in a moral sense. They hold a "developmental" or "gradualist" view of life's beginning (Ford, 1988; McConnick, 1991; Warren, 1997; Green, 2001; Shannon, 2001). They do not deny that the early embryo is alive, and has the biological potential to become a person. Nevertheless, they believe that other features are needed for the full and equal protection we normally accord children and adults, and that these features only develop gradually across the full term of gestation. These features include such things as bodily form, and the ability to feel or think. The

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early embryo, they maintain, does not have these features or abilities. They note also that the very early embryo lacks human individuality, since it can still undergo twinning at this early stage, and two separate embryos with distinct genomes can fuse to become a single individual (Strain *et al.*, 1998). The very high mortality rate of such embryos (most never implant) also reduces the force of the argument from potentiality (Hardy *et al.*, 2001; Norwitz *et al.*, 2001). Those who hold this view do not agree on the classes of research that warrant the destruction of embryos, but most support some form of hES cell research. Their reasoning is that, although the early embryo may merit some respect as a nascent form of human life (Lebacqz, 2001), the lives and health of children and adults outweigh whatever claim it possesses.

Each individual faced with involvement in hES cell research must arrive at his or her own answer to this first question. Legislators and others must also wrestle with these issues. Because American law (and the laws of most other nations) does not regard the early embryo as a person meriting the legal protections afforded to children and adults, it is hard to see how one can justify legal or regulatory prohibitions on privately-financed hES cell research or clinical applications. Such prohibitions would interfere with individual liberty, on grounds inconsistent with the reduced status of the embryo shown elsewhere in the law. However, because publicly-funded research rests on more narrowly political considerations, including the way that a majority chooses to spend public funds, it may be expected that public support for hES cell research will depend on how a majority of citizens answer this first question.

SHOULD WE POSTPONE HUMAN EMBRYONIC STEM CELL RESEARCH?

Some who oppose the destruction of human embryos maintain that hES cell research should be deferred, at least until science provides a better view of the likely benefits of adult stem cell research. They maintain that this research direction is as promising as hES cell research, and they argue that the moral acceptability of the alternative justifies any delay in the availability of therapies (Doerflinger, 1999). Others have argued that the scientific uncertainty, and ethically controversial nature of hES cell research, warrants a moratorium on it in both the private and the public sectors (President's Council on Bioethics, 2002). Scientific studies using adult stem cells continue to be equivocal. They have sometimes supported, and at other times undermined, claims to the plasticity of adult stem cells, their ability to immortalize, and their usefulness (Pearson, 2002). This raises the question of whether it is justified to delay the development of therapies and cures for children and adults to protect embryos and respect the sensitivities of those opposed to embryo research. Many people feel that such a delay is not warranted, and that it is scientifically and morally preferable to keep multiple pathways to stem cell therapies open. In the words of the National Research Council, "The application of stem cell research to therapies for human disease will require much more knowledge about the biological properties of all types of stem cells" (National Research Council, 2001).

CAN WE BENEFIT FROM OTHERS' DESTRUCTION OF EMBRYOS?

It might seem that a negative answer to the first question ends discussion. If it is morally wrong to destroy a human embryo, what could justify the use of cells derived from its deliberate destruction for other people's benefit? However, research or therapy has many steps, not all of which involve the destruction of embryos. This raises the question of whether downstream researchers, clinicians, or patients may use the stem cell lines that others have derived. Ethically, this is a question of whether we can ever benefit from deeds with which we disagree morally, or regard as morally wrong (Kaveny, 2000; Green, 2002).

This question arises partly because most embryos used to produce stem cell lines are left over from infertility procedures. Couples using in vitro fertilization (IVF) routinely create more embryos than can safely be implanted. There are hundreds of thousands of these embryos in cryogenic freezers around the country, and around the world (Weiss, 2003). Since few frozen embryos are made available for adoption (Stolberg, 2001), most of these supernumerary embryos will be destroyed. In 1996, British law mandated the destruction of 3600 such embryos (Ibrahim, 1996). This destruction continues, regardless of whether some embryos are diverted to hES cell research.

Why is it morally wrong to benefit from others' wrongdoing? One answer is that by doing so, we encourage similar deeds in the future. This explains why we are morally and legally prohibited from receiving stolen goods, or why it may be wrong to benefit from research produced by scientists who choose to ignore human–subject constraints (Green, 2002). However, it seems less objectionable to benefit from others' wrongdoing when their deeds are independently undertaken, when our choices are not connected to theirs, and when the choices do not encourage wrongful deeds. For example, few people would object to using the organs from a teenage victim of a gang killing to save the life of another dying child. The use of such organs benefits one young person, and does not encourage teen violence. Similar logic might apply to stem cell research, using spare embryos remaining from infertility procedures. A downstream researcher, clinician, or patient may abhor the deeds that led to the existence of an hES cell line, including the creation and destruction of excess human embryos in infertility medicine. But nothing that a recipient of an hES cell line chooses to do is likely to alter, prevent, or discourage the continuing creation or destruction of human embryos,

or make the existing lines go away. Those who use such embryos also know that if they refuse to use an hES cell line, they could forego great therapeutic benefit. A researcher will fail to develop a lifesaving or health-restoring therapy. A clinician's decision may risk a patient's life. People in this position will struggle with the question of whether it is worthwhile to uphold a moral ideal, when doing so has no practical effect, and when it threatens injury to others.

Religious views on the question of whether one may ever benefit from others' wrongdoing are diverse. The Roman Catholic moral tradition, despite its staunch opposition to complicity with wrongdoing, presents different answers, including some that permit one to derive benefit in particular cases (Miller, 1967; Ashley and O'Rourke, 1989; Green, 2002). This suggests that some researchers, clinicians, or patients who morally oppose the destruction of human embryos might ethically use hES cell lines derived from embryos otherwise slated for destruction.

It is noteworthy that in his August 2001 address to the nation, President George W. Bush adopted a conservative version of the position that allows one to benefit from acts one morally opposes. Stating his belief that it is morally wrong to kill a human embryo for others' benefit, the president nevertheless permitted the use of existing stem cell lines on the grounds that the deaths of the embryos had already occurred (Bush, 2001). Presumably, the president believed that it would not encourage further destruction of embryos to permit this use. The president did not go so far as to permit the use of lines derived from embryos certainly slated for destruction, because their progenitors had chosen never to use them. However, in April 2009, the National Institutes of Health, did just this. Implementing an Executive order issued in March by President Barack Obama, the NIH authorized funding for the use, but not the derivation of new hES cell lines from embryos remaining from infertility procedures (National Institutes of Health, 2009).

CAN WE CREATE AN EMBRYO TO DESTROY IT?

A fourth question takes us into even more controversial territory. Is it ever morally permissible to create an embryo deliberately to produce a stem cell line? This was done in the summer of 2002 at the Jones Institute in Norfolk, VA (Kolata, 2001). Those in favor of this research defend it on at least two grounds. First, they say that in the future, if we seek to develop stem cell lines with special properties, and perhaps closer genetic matches to tissue recipients, it will be necessary to produce stem cell lines to order using donor sperm and eggs. Second, they argue that it is ethically better to use an hES cell line created from embryos that have been produced just for this purpose, with the full and informed consent of their donor progenitors, than to use cell lines from embryos originally created for a different, reproductive purpose.

Those who believe the early embryo is our moral equal oppose the deliberate creation of embryos for research or clinical use. They are joined by some that do not share this view of the embryo's status, but who believe that it is morally repellant to deliberately create a potential human being only to destroy it (Annas *et al.*, 1996). Such people argue that this research opens the way to the "instrumentalization" of all human life, and the use of children or adult human beings as commodities. Some ask whether such research does not violate the Kantian principle that we should never use others as "a means only" (Green, 2001).

On the other side of this debate are those who believe that the reduced moral status of the early embryo permits its creation and destruction for lifesaving research and therapies (National Institutes of Health, 1994; Davis, 1995). The proponents of this research direction ask why it is morally permissible to create supernumerary embryos in IVF procedures to help couples have children, but morally wrong to do the same thing to save a child's life. They are not persuaded by the reply that the status of the embryo is affected by its progenitors' intent, and that it is therefore permissible to create excess embryos for a "good" (reproductive) purpose, but not for a "bad" (research) purpose. They point out that the embryo is the same entity, and its status should not depend on its progenitors' intentions. Thus, we do not ordinarily believe that a child's rights are dependent on its parents' intent or degree of concern (Parens, 2001). They conclude that it is not parental intent that warrants the creation of excess of embryos in such cases, but the embryos' lesser moral status and the likelihood of significant human benefit from their use. These same considerations, they believe, justify deliberately creating embryos for stem cell research.

SHOULD WE CLONE HUMAN EMBRYOS?

A fifth question is whether we are willing to support human cloning for stem cell research. This question arises in connection with a specific stem cell technology known as "human therapeutic cloning." It involves the deliberate creation of an embryo by somatic cell nuclear transfer technology (cloning) to produce an immunologically compatible (isogenic) hES cell line (Lanza *et al.*, 2000).

Immune rejection could occur if the embryo used to prepare a line of hES cells for transplant does not share the same genome as the recipient. This would be the case whether the cell line was created from a spare embryo, or from one made to order. Therapeutic cloning offers a way around this problem. In the case of a diabetic child, the mother could donate an egg whose nucleus would then be removed. A cell would be taken from the child's body, and its nucleus inserted into the egg cytoplasm. With stimulation, the reconstructed

cell would divide, just like a fertilized egg. If the resulting embryo were transferred back to a womb, it could go on to birth and become a new individual—a clone of the child. But in therapeutic cloning, the blastocyst would be dissected and an hES cell line prepared. Growth factors could be administered to induce the cells to become replacement pancreatic cells for the child. Because these cells contain the child's own DNA, and even the same maternal mitochondrial DNA, they would not be subject to rejection. Research has also shown that the small amount of alien RNA from the mitochondria of an egg other than the mother's would probably not provoke an immune rejection (Lanza *et al.*, 2002).

Although this is a very promising technology, it raises a host of novel ethical questions. One is whether the embryonic organism produced in this way should be regarded as a "human embryo" in the accepted sense of that term (Nature, 2001). Those who believe that "life begins at conception" tend to answer this question affirmatively, even though cloned "embryos" are not the result of sexual fertilization (Doerflinger, 2003). They base their view on the biological similarities between cloned and sexually produced embryos, and on the argument that both have the potential to become a human being. Nevertheless, the very high mortality rate of cloned embryos suggests significant biological differences from sexually produced embryos. Furthermore, if the embryos' status rests on their potential, this potential is more than significantly reduced; in an era of cloning, some degree of potentiality attaches to all bodily cells.

The promise of this technology rests on the ability to make stem cell lines "to order" for a specific patient. If you hold the view that the cloned organism is morally equivalent to a human embryo, therapeutic cloning research and therapies again raise the question of whether it is morally permissible to create an embryo deliberately to destroy it.

Another question often raised in this context is whether therapeutic cloning will create an enormous demand for human eggs. If it does, some believe this may create substantial problems of social justice, since collecting these eggs may involve hundreds or thousands of women, many of whom are likely to be poor women of color attracted by the financial rewards of egg donation (Dresser, 2001; Holland, 2001). Those who discount this problem offer several arguments. They point out that even if many eggs are needed for therapeutic cloning procedures, they are likely to be provided by relatives of patients. This reduces the magnitude of a market in oocytes. Others observe that therapeutic cloning is likely to be "transitional research" (Chief Medical Officer's Expert Group, 2000). As such, it may eventually be replaced by direct somatic cell reprogramming, eliminating the need for eggs altogether.

Finally, there is a moral question specific to cloning itself. The more scientists are able to perfect therapeutic cloning, the more likely it is that they will sharpen the skills needed to accomplish reproductive cloning, which aims at the birth of a cloned child. There is a broad consensus in the scientific and bioethics communities that, for the foreseeable future, the state of cloning technology poses serious health risks to any child born as a result of it (Jaenisch and Wilmut, 2001). There are also serious, unresolved questions about the psychological welfare of such children (National Bioethics Advisory Commission, 1997). Finally, there is the possibility that embryos created for therapeutic cloning research might be diverted to reproductive cloning attempts. All these concerns raise the question: do we really want to develop cloning technology for the production of isogenic stem cells, if doing so hastens the advent of reproductive cloning (Weiss, 1999)?

In 2001, and again in 2003, the US House of Representatives answered "no" to this question, and passed a bill introduced by Rep. James Weldon that banned both reproductive and therapeutic cloning. Similar bills have been introduced in the Senate (Stolberg, 2001, 2003). Although the Senate initiatives have been stalled for some time, this may change (New York Times, 2003). If a Senate bill passes, therapeutic cloning research and therapies will be outlawed in the United States. Similar prohibitions are either in effect, or being considered for passage in continental Europe (BBC News, 2006). This would leave only a relatively small number of countries, including Great Britain, Israel, and China, in which such research would be allowed (Israel Academy of Sciences and Humanities, 2001).

Those who oppose these prohibitions believe that therapeutic and reproductive cloning research can be decoupled. They observe that strict regulations and governmental oversight, of the sort provided in Great Britain by the Human Fertilisation and Embryology Authority, make it unlikely that embryos produced for therapeutic cloning will be diverted to reproductive purposes. They also point out that several researchers or groups with minimal qualifications in cloning research have announced their intent to clone a child, or have even tried to do so. Such attempts are likely to continue, regardless of whether therapeutic cloning research is banned. As a result, a ban on therapeutic cloning will not protect children, and will only have the negative effect of interrupting beneficial stem cell research

DOES INDUCED PLURIPOTENT STEM CELL RESEARCH ELIMINATE THE ETHICAL QUESTIONS SURROUNDING HUMAN EMBRYONIC STEM CELL RESEARCH?

In late 2007, teams led by Yamanaka in Japan and Thomson in the US announced success in the use of gene transfer technology to produce pluripotent stem cell lines (Takahashi *et al.*, 2007; Yu *et al.*, 2007). Some believe that this development has ended debates about the appropriateness of using hES cell lines (Krauthammer, 2007). If we can directly manipulate somatic cells, why should we engage in the ethically controversial activity of destroying embryos? However, there are scientific and ethical issues that challenge this view.

On the scientific side, there is a question of whether iPS cell lines will prove suitable for use in human transplant and cell regeneration therapies. Current iPS cell lines exhibit high rates of tumorigenicity in mice, a result of the use of retroviral vectors to carry pluripotency-inducing transcription factors, including the cancer-related factor c-Myc (Hyun *et al.*, 2007). Direct up-regulation of the relevant genes and replacement of c-Myc may eventually overcome this problem, but until this is accomplished, iPS cell lines cannot ethically be used for human transplant purposes. They may be of value, however, for the creation of model lines of disease-related cells.

Also questionable is the widespread assumption that inducing pluripotency in somatic cells raises no ethical questions. It is true that protecting every body cell from gene manipulation would seem absurd. But is this so if the genetic manipulation of a cell renders it capable of eventual development into a human being? The work of Nagy and others shows that ES cells, when inserted into tetraploid embryos, are able to develop the placental material needed for further development (Nagy *et al.*, 1990, 1993; Magill and Neaves, 2009). This possibility raises complex ethical questions. Is potentiality morally relevant if it is accompanied by such intensive technical interventions? IVF or cloned embryos also require intensive technical interventions. Those who favor iPS cell research, but oppose the use of IVF or cloned embryos as sources of pluripotent stem cells will have to examine the consistency of their views. If it is acknowledged that the potentiality for full human development, however assisted, confers moral status on an entity, then iPS research may not be the panacea that hES cell research opponents believe it to be.

WHAT ETHICAL GUIDELINES SHOULD GOVERN HUMAN EMBRYONIC STEM CELL, THERAPEUTIC CLONING, AND INDUCED PLURIPOTENT STEM CELL RESEARCH?

Mention of the need to prevent the diversion of cloned embryos to reproductive purposes raises the larger question of what guidelines should apply to the conduct of hES cell, therapeutic cloning, and iPS research. In August 2000, the US National Institutes of Health released a series of guidelines for hES cell research that never went into effect, because they were largely preempted by President Bush's decision to limit hES cell research to existing cell lines (National Institutes of Health, 2000). Guidelines have also been developed by the Chief Medical Officer's Expert Group in Great Britain (Holland, 2001), by private ethics boards at the Geron Corporation (Geron Advisory Board, 1999) and Advanced Cell Technology (Green *et al.*, 2002) in the US, by committees of the National Research Council (Committee on Guidelines for Human Embryonic Stem Cell Research, National Research Council 2005; National Research Council and Institute of Medicine of the National Academies, 2007, 2008), and by the International Society for Stem Cell Research (ISSCR, 2006). These various recommendations share several features.

Escro Review

First, there is agreement that, in addition to the usual scientific and review of research proposals by an Institutional Review Board, it is appropriate that there be another layer of review provided by an Embryonic Stem Cell Research Oversight (ESCRO) committee. ESCROs are needed to review protocols involving special issues, such as the transplanting of hES cell materials into human subjects, and research that generates chimeric animals using human cells. This research is particularly sensitive, because of the unknown effects of differentiation and integration of the human cells into nonhuman animal tissues, especially cerebral cortex or germline. There are special cautions about breeding chimeric animals in which human pluripotent cells have been introduced into germline tissues (Krauthammer, 2007).

Donor and Procurement Issues

Second, because hES cell, therapeutic cloning, and iPS cell research require a supply of human gametes and embryos, any of which raise sensitive issues of moral status, steps must be taken to elicit the informed consent of donors, to protect their privacy, and to minimize any risks to which they might be subject. Because non-embryo-derived iPS cells are derived from human material, their derivation is covered by existing IRB regulations concerning review and informed consent, and no ESCRO committee review may be necessary. Nevertheless, the possibility of the use of such cells in chimeric animals and special transplantation issues probably recommend ESCRO review. To aid researchers, both the NRC and ISSCR have offered the outlines of model consent documents for these purposes (Hyun *et al.*, 2007; Krauthammer, 2007).

In general, informed consent requires that donors fully understand the nature of the research being undertaken (for example that it may require the destruction of an embryo), and that they explicitly consent to that research. Whenever possible, they should be given the option of agreeing to some forms of hES cell research, but not others (e.g. the creation of stem cells, but not cloned embryos). It is morally impermissible to elicit somatic cells, sperm, eggs, or embryos for the production of stem cell lines without informing donors that an immortalized, pluripotent cell line might result that could be widely used in research or therapeutic applications. If there are likely to be commercial benefits flowing from the research, donors must also be informed of this, and their

rights (if any) in such benefits should be clearly specified. If the research involves therapeutic cloning, both egg and somatic cell donors must be informed that a cloned embryo and a cell line with the egg donor's mitochondrial genetic material and a somatic cell donor's nuclear DNA will result. IPS cell somatic cell donors should also be informed about the developmental and commercial possibilities of their cellular materials. Special questions also arise about the use of somatic cells, embryos, or sperm whose provenance is uncertain, including embryos created with sperm whose donor is unknown (Sugarman and Siegel, 2008).

In conducting research, efforts should be made to preserve donor privacy, by removing identifying information from gametes, embryos, and cell lines, and keeping this information apart in a secure location. In view of the controversy surrounding much of this research, donors can be subjected to harassment or embarrassment if their association with the research is revealed without their consent.

Therapeutic cloning research raises the special issue of ovulation induction. This is an invasive medical procedure, with both known and undetermined risks (Paulson, 1996; Rossing *et al.*, 1996). Not only must egg donors be informed of these risks, but steps also must be taken to preserve the voluntary nature of their consent. This includes avoiding undue financial incentives. It also includes preventing them from being pressured into producing excess eggs or embryos for research in return for discounts on infertility services (Cohen, 2001). Although it is unreasonable to expect even altruistically-motivated donors to undergo the inconvenience and risks of these procedures without some form of compensation, payment should not be so high as to lead a donor to ignore the risks involved. For Advanced Cell Technology's therapeutic cloning egg donor program, its Ethics Advisory Board set a payment level similar to that established for reproductive egg donors in the New England area. Payments were also prorated for the degree of participation in the program, to allow donors to drop out at any time. Levels of stimulatory medications were maintained on the low side of current regimens, and payments were never attached to the number of eggs harvested. Additional protections ensured that donors had the educational level and background needed to appreciate the risks involved. A study monitor was employed to ensure donors' consent was free and informed.

Research Conduct

Guidelines also apply to the overall conduct of research. These include the requirement that no embryo used in hES cell or therapeutic cloning research be allowed to develop beyond fourteen days. This limit is based on the substantial changes that occur at gastrulation, which marks the beginning of individualization and organogenesis (O'Rahilly and Müller, 1992). Supervision and accountability of all staff and scientists involved in this research to prevent any diversion of gametes or embryos to reproductive purposes is also required.

Transplantation Research

If and when hES cell lines become available for research in transplant therapies, researchers and institutional review boards will have to address ethical questions raised by their use. For example, if such lines were cultured on mouse or other feeder layers, as almost all of the federally-approved lines were, there will be a safety question regarding the possible introduction of exogenous retroviruses or other pathogens into the human population. Basing their judgments on adequate preliminary animal studies, researchers and oversight bodies will have to assess the risks of rejection, including graft-versus-host disease. Issues surrounding the tumorigenicity of hES and iPS cells will also have to be resolved. Experience with fetal cell transplants for Parkinson's disease shows that cells can behave in unexpected ways when transplanted into the human body (Kolata, 2001). None of these problems are insurmountable, but their existence constitutes an argument for continued research in this area under careful ethical oversight.

SUMMARY

Fully answering all of the questions I have raised would require an ethical treatise. However, in my view, the moral claims of the very early embryo do not outweigh those of children and adults that can be helped by hES cell and therapeutic cloning technologies. While iPS cell research is promising, reliance on it should not, in my view, replace the use of hESC lines produced through proven techniques, and that are currently available. I also do not believe that therapeutic cloning research will lead to reproductive cloning, which we should take firm steps to forbid. I recognize that others may disagree with these conclusions. As our debates move forward, continuing dialogue about these questions, and clearer scientific research results will bring us closer to a national consensus on these issues.

KEY WORDS

Bioethics The study of the norms of conduct that should govern research and clinical applications of biomedical technologies.

Stem cells Self-renewing (immortalized), unspecialized cells that can give rise to multiple types of all specialized cells.

Therapeutic cloning The deliberate creation of a human organism by nuclear transfer (cloning) technology in order to produce an embryo that can be used to create a stem cell line that will not provoke an immune response and rejection.

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Chapter 67

Stem Cell Research: Religious Considerations

Margaret A. Farley

INTRODUCTION

Religious traditions have endured in large part because they help people to make sense of their lives. Major religious traditions become major because they offer some response to the large human questions of suffering and death, hope and transcendence, history and community, as well as the everyday issues of how we are to live together with some modicum of harmony and peace. Adherents of traditions of faith generally experience their shared beliefs and practices, not as irrational, but as part of the effort of reason to understand the actual and the possible in human life, and all that is around it. Although religious sources of insight may reach beyond empirical data and logical reasoning, they need not ultimately do violence to either. When we ask profound questions such as how humans ought to reproduce themselves, or how extreme suffering can be alleviated, or whether the meaning of the human body changes when we exchange its parts, we can be helped by looking to the source and the substance of the attitudes, beliefs, and practices which animate people's lives.

Of course, when we look for guidance in the faith of believers, we do not find univocal voices—not from believers in general, and not from believers within particular traditions. Roman Catholics will often not agree with spokespersons from the United Church of Christ; many Jews will not agree with many Catholics about abortion; and so on. But the division within faith traditions today is often wider than between or among them. So, there is more than one view regarding stem cell research among Roman Catholics; more than one among the different strands of Judaism; more than one among Muslims. Such disagreements do not make religious voices useless for societal and religious discernment, no more than disagreements among scientists render scientific research and the voices of scientists useless.

This chapter aims to review religious perspectives on stem cell research. It is not possible here to survey every religious or theological argument for and against stem cell research. Something of an overview is presented, and then particular issues of concern articulated by representatives of some religious traditions are examined.

MAPPING THE TERRAIN

Sources for Stem Cells

The major disputed questions surrounding stem cell research are focused, not on stem cells as such, but on the sources from which stem cells are derived. Hence, debates rage regarding the moral status of human embryos and the permissibility of taking stem cells from already dead fetuses. Religious thinkers who consider the human embryo sacrosanct from its inception will consistently oppose the extraction of embryonic stem cells as a form of killing (of embryos). Those who do not appraise the value of an embryo as being on a par with that of a human person are much more willing to favor embryo research in general, including the taking of stem cells for research. Still others who emphasize the ambiguity of the moral status of embryos are likely to weigh the advantages of embryo cell research over the possible violations of the embryo as an entity in itself. All religious traditions recognize an aborted fetus as a cadaver; hence, the derivation of stem cells from the gonadal ridge of dead fetuses may be as permissible as the harvesting of tissues or organs from any human cadavers. Nonetheless, representatives of traditions that prohibit abortion worry about complicity with, and support of, the moral evil of abortion when stem cells are derived from electively aborted fetuses.

Alternative sources for human stem cells are championed by those who are opposed to embryo stem cell and fetal stem cell research. The least objectionable sources often cited are umbilical cord blood, placentas, amniotic fluid, and adult somatic stem cells (extracted, for example, from bone marrow). Other sources considered in 2005 by the President's Council on Bioethics include embryos that are already dead but have some remaining normal cells; blastomeres biopsied from living embryos without destroying the embryos; deliberately engineered biological artifacts that are not embryos, but

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function like embryos minus any potential to develop into a person. A compromise source (although still controversial) is lines of cells derived from stem cells that have already been harvested (that is, the life of the original embryo has already been taken, and no new moral agency is involved in the killing of more embryos). Despite significant progress on some of these alternative sources—especially adult tissues and cells—there remain serious scientific, religious, and ethical problems.

Scientists note that research on adult sources is still less advanced than research on embryonic cells, and in any case, many scientists argue that it is better to proceed on all fronts. The use of embryo cadavers raises new ethical questions about in vitro fertilization and the cryopreservation of embryos (about half of which die when thawed). Removing cells from living embryos is not without risk to the embryos. The creation of biological artifacts can involve cloning (which, even when it is for research, not reproduction, raises another set of ethical issues). Moreover, the artifacts may be "functionally equivalent" to embryos in ways that fail to settle all questions of their moral status—that is, maybe they should be respected in the same way as embryos are respected, since their incapacity to become human persons may be overcome at some point in the further development of reproductive technologies. In addition, a move to "create" these artifacts crosses another moral boundary—manufacturing entities precisely for research, as opposed to simply using "spare" ones—that is troubling for many people. Finally, the proposed use of already existing embryonic cell lines encounters the same objections of complicity with moral evil that is involved in the use of cells taken from aborted fetuses. For those who take seriously all of these objections to otherwise promising alternative sources for human stem cells, the alternatives do not appear as yet to resolve ongoing deep-seated scientific, ethical, or religious concerns.

Other issues complicate moral assessments of the sources of stem cells, and the aims of stem cell research. For example, almost all religious thinkers argue that some form of respect is due to embryos, even if it is not the same form of respect required for human persons. Yet there is lack of clarity about what respect can mean when it is aligned with the killing of the respected object. It is not difficult to get minimal consensus on prohibitions against buying and selling human embryos or on safeguards of informed consent from the donors of embryos for research. For many religious believers, it makes a difference that these embryos are "spare" embryos, left over from in vitro fertilization procedures, and destined to be discarded if they are not used in research. From other religious perspectives, however, this fact in itself does not lift the prohibition against killing them.

Questions of Justice

Quite another kind of ethical issue is raised by people from almost all religious traditions. This is the issue of distributive justice. Who will be expected to be the primary donors of embryos or aborted fetuses? Will gender, race, and class discrimination characterize the whole process of research on stem cells? And will the primary goals of research be skewed toward profit rather than toward healing? Who will gain from the predicted marvelous therapeutic advances achieved through stem cell research—the wealthy but not the poor? The powerful, but not the marginalized? What will be the overall results for respect for human persons if human embryos and stem cells are commodified, wholly instrumentalized, and in some ways trivialized?

All of these questions are questions for secular philosophical, scientific, and medical ethics, as well as for religious ethics. When they are identified by religious and theological thinkers, however, they are always lodged in the larger questions with which religious traditions are concerned, and they almost always appeal to sacred texts and faith community traditions, teachings, and practices, as well as to secular sources and forms of moral reasoning.

Religious Beliefs and Ethical Questions

Anchoring religious concerns for the moral status of the human embryo, the nature of moral evil and immoral complicity, and questions of distributive justice, are beliefs about the nature and destiny of human life, the meaning of bodily existence, the interaction between divine and human in the progress and healing of creation, the importance of consequences in moral reasoning along with the importance of religious laws and ontologically based norms, the basic equality of all human people before God, and so forth. Even when there is no direct line between the most profound beliefs of a person and the answer to a particular moral question, such a belief makes a difference for moral discernment and conviction. There are no world religions today that oppose all human intervention in "nature," yet all religions recognize some limitations on what humans may do—either to themselves or to other created beings. All consider humility before concrete reality an important antidote to arrogance or pride (and in the wake of ecological disasters, all now take account of the risks of some interventions made only for the sake of the flourishing of present, but not future, human generations). No religions favor illness over health, or death over life, yet each has a perspective in which health may not be an absolute value, and death may sometimes even be welcomed. In the present context of stem cell research, there is no religious tradition that does not take the beneficial results promised by such research seriously.

Hence, positions of religious believers regarding stem cell research tend to be highly complex. Each tradition needs to be understood in its complexity and its diversity. This chapter cannot track all of this, but it can try to show how some beliefs are coherent, whether or not others can agree on them in the same tradition, or across faiths and cultures.

PARTICULAR TRADITIONS: AN OVERVIEW

A basic understanding of positions taken by religious scholars and spokespersons in mainline traditions is, fortunately, available to some extent in the testimony and writings that have appeared in relation to the public debate on stem cell research. We have here, of course, just the proverbial tip of the iceberg. Nonetheless, these documents and essays point us to both the concrete moral positions being debated, and the larger rationales behind them. In brief summaries, then, I survey some of these positions and rationales. Following this, I focus on specific lines of argument taken by representatives of the Roman Catholic tradition. I do this not to privilege this tradition, but because its articulation of arguments is more prolific than most; it claims to be trying to be persuasive in a secular public sphere; its central positions are representative of strands of other religious, as well as philosophical traditions; and it offers an ongoing critical conversation on both sides of the stem cell debate.

Hinduism and Buddhism

Representatives of the Hindu and Buddhist traditions have not been major players in the contemporary debate about stem cell research, at least not in the United States. There are historical reasons for this no doubt, including the fact that neither is as yet a majority religion in the West. Yet there is much in both of these traditions that may, for their adherents, be relevant to issues of stem cell research. Given the complexity of the traditions, it remains for their scholars and representatives to render accessible to outsiders the foundational beliefs and moral concerns that may shed light on such issues. Briefly, however, some elements in these faith traditions can be noted.

Both Hinduism and Buddhism incorporate beliefs in reincarnation. This complicates issues of what can be done with human embryos. According to some Hindu traditions, human life begins prior to conception; the "soul" may be present even in sperm, or it may be in some other life form before and after its human existence. Human reincarnation, however, offers a unique opportunity to influence the future of an individual. Great caution must be taken whenever actions are considered that may destroy this opportunity. On the other hand, for Hindus, a belief in reincarnation is also tied to motivation to be compassionate towards others. Good actions that will change the course of suffering for oneself and others are exhorted, not only so that one can advance morally (and thereby improve one's karma), but because other people are worthy of beneficent deeds. Although the forms of compassion include nonviolence, there is in Hinduism also a broad tradition of sacrifice, wherein one human life can be taken for the sake of a higher cause. It is not impossible that these convictions provide a rationale for embryonic stem cell research. As one example of this, in 2001 the Hindu Endowments Board of Singapore expressed willingness to accept the extraction of stem cells from human embryos in their earliest stages, as long as this did not go beyond 14 days of embryonic development.

Buddhists are divided in their opinions about human embryonic stem cell research. For some, the precept against harming any living thing is determinative, ruling out the extraction of stem cells from, and the concomitant destruction of, embryos. Yet other Buddhists appeal to another element in Buddhism that offers a possible rationale for embryonic stem cell research. Buddhism's goal of self-transcendence through a process of self-forgetting undergirds a requirement for compassion, one of the Fourfold Holy Truths of Buddhism. In Mahayana Buddhism, the ideal is the Bodhisattva, one who achieves self-emptying, but then returns to help those in need. Again, here is a possible rationale for embryonic stem cell research. Moreover, some Buddhists believe that a soul has some choice as to where it will be incarnated. It has even been suggested that souls will not elect to embed in "spare" embryos that will only be destroyed. Hence, early embryos may not ever have a potential to become a human person, and can therefore be used for some other purpose.

Whether such considerations are of overall significance in decisions to pursue embryonic or fetal stem cell research or not, remains to be seen. At the very least, it can be said that they represent large concerns for the value of human life—whether at its inception, or in response to later injury and illness.

Islam

Like other world religions, there are many schools of thought among Muslims. There are also multiple sources for moral guidance, including the Qur'an and its commentaries, the hadiths (a second source of moral indicators supplementary to the Qur'an), Muslim philosophies that range from a form of ontological and moral realism to a version of divine command theory, Sharia (formulations of Islamic law), and centuries of juridical literature. Testifying before the US National Bioethics Advisory Commission in 1999, the Islamic scholar Abdulaziz Sachedina attempted to provide some general insights from the tradition, taking into account the diverse interpretations of major Sunni and Shi'i schools of legal thought. His analysis of various texts led him to infer guidelines for stem cell research from the rulings of the Sharia on fetal viability and the moral status of the embryo.

Islamic traditions have given serious attention to the moral status of the fetus, and its development to a particular point when it achieves human personhood with full moral and legal status. The early embryo has been variously valued in different eras, depending on the information available from science. Throughout the history of legal rulings in this regard, however, a developmental view of the fetus—according to a divine plan—has been sustained. This founds the view that the moral status of personhood is not achieved

at the earliest stages of embryonic life, but only after sufficient biological development has taken place—the kind of development that includes a recognizable human anatomy and the possibility of "voluntary" movement. Most Sunni and some Shi'ite scholars therefore distinguish two stages in pregnancy. The first stage, "pre-ensoulment," is human biological life, but not yet human personal life. It is only after the fourth month (120 days, or the time of quickening) that the "biological human" becomes a "moral person." This is to say that, at this stage of development, the fetus achieves the status of a moral and legal person. In the first stage, the biological entity is to be respected, yet abortion is allowed for grave reasons. In the second stage, killing the fetus is homicide. The conclusion to be drawn from this is that there is room for early embryonic stem cell extraction, without violating divinely given laws or the embryo itself. In Islam, therefore, there are strong elements of the tradition that affirm embryonic stem cell research.

Judaism

In the twenty-first century, Orthodox, Conservative, and Reform Jews take different positions on many issues of applied ethics. These views are not easily specified when it comes to stem cell research. What is possible here is to present the opinions of some Jewish scholars, with the caution that not all views are represented. In the context of debates about stem cells, Reform Judaism may be the clearest supporter of this research, even when it entails the derivation of stem cells from human embryos, or from aborted fetuses. Jewish law does not give legal status to the fertilized ovum outside a mother's womb. When the embryo is inside a uterus, it has legal status but not that of a human person. In general, however, this strand of Judaism favors the use only of so-called spare embryos, those that will otherwise be discarded.

Rabbi Elliott Dorff has identified theological assumptions useful for understanding a Jewish response to stem cell research. These include the following: moral discernment (of what God wants of God's people) must be based on both Jewish theology and Jewish law; all human beings are created in the image of God and are to be valued as such; human bodies belong to God and are only on loan to the individuals who have them; human agency is important in responses to human illness, so that both "natural" and "artificial" means are acceptable in overcoming disease. Indeed, there is a duty to develop and use any therapies that can help in the care of the human body. Yet, because humans are not, like God, omniscient, humility and caution are essential, especially when human science and technology press to the edges of human knowledge.

There are grounds in Jewish law and theology for permitting the derivation of stem cells, both from aborted fetuses and from human embryos. Just as abortion (although generally forbidden) can be justified for serious reasons, so also can the use of fetuses for important research. A fetus in utero is considered not as a human person but as the "thigh of its mother." One is not allowed to amputate a part of one's body (in this case, one's thigh) except for good reasons (for example, to remove a gangrenous limb, save the life of a mother, or remove other serious risk, and perhaps remove a fetus that is genetically malformed or diseased). When an abortion is thus justified, the abortus may be used as a source of stem cells.

Embryos can also be legitimate sources for stem cells extracted for purposes of research. During the first 40 days of gestation, in utero embryos (and fetuses) are only like "water." They are "non-souled," with only a liminal status. Although abortion during this time is permitted only for good reasons, the situation is different when embryos are not in the womb. Here there is no potential for embryos to develop into human persons; hence, they may be discarded, frozen, or used for promising research. Both Rabbi Dorff and Jewish scholar Laurie Zoloth argue that the duty to care for human bodies, the duty to heal, may undergird not just permission to pursue research on stem cells, but an ethical duty to do so.

The Jewish tradition places all of these considerations in a wider context of responsibilities to community, fairness in distribution of benefits and burdens, and caution about the connections of stem cell research to invidious programs of eugenics. Ethical norms that safeguard the common good, as well as the good of individuals, provide boundaries for the development and future uses of stem cell research; they focus it, perhaps obligate it, but do not forbid it.

Christianity

The diversity and pluralism to be expected in Christian responses to stem cell research mirrors to some extent the historical institutional divisions within Christianity. From the early schisms between East and West to the great Reformations in the West and the subsequent proliferations of Protestant denominations, to the Anglican and Roman Catholic Church traditions, to the rise of new forms of Pentecostalism in the world's South, Christians have diversified in their beliefs, and in their moral convictions and practices. There remains some important commonality in basic doctrines, largely expressed in creeds, and these are not irrelevant to issues of stem cell research. Examples include belief in divine revelation, particularly through sacred scripture; reconciliation made possible between humans and God through Jesus Christ; affirmation of the goodness of creation despite the damage sustained as the result of moral evil or sin; acceptance of the importance of human agency (in practice if not always in theology); a call to unconditional love of God, and love and service of neighbor; a basic view of human equality; and the obligation to promote justice. Theological anthropologies vary importantly in terms of understandings of freedom and grace, virtue and sin.

Strands of Christian moral theologies diverge when it comes to the basis and force of moral norms—depending primarily either on God's command or God's will manifest in creation itself, and holding to absolute moral norms or only prima facie norms relativized according to circumstances, consequences, or priorities among aims. Law and gospel are important to each Christian tradition, although the emphasis on one or the other may vary.

Articulating an Eastern Orthodox view of embryonic stem cell research, Demetrios Demopulos begins with concern for the alleviation of suffering. "Medical arts" are to be encouraged, with the proviso that they be practiced as gifts from God ordered to divine purposes. Created in the image and likeness of God, people are destined for participation in the life of God—hence the telos of the human person is referred to as theosis or "deification." People are authentic insofar as they struggle to grow into this life; they remain "potential" persons until this is achieved. But even zygotes are potential persons in this sense; that is, they, too, are in a developmental process that ends in deification. Hence, human life is sacred from the beginning, and at every step along the way, entitled to protection and the opportunity to seek its destiny. Even though it is not yet a person, an embryo should not be used for, or sacrificed in, research. Correlatively, practices of in vitro fertilization that yield "surplus" embryos which will be discarded cannot be condoned.

Although the development of alternative techniques and sources is preferable, Demopulos allows the use of already-harvested embryonic stem cells for research. Wishing that something had not been done will not undo it, he maintains. The compromise position, then, of continuing research on available cell lines is accepted, so long as the research does not violate other norms of justice (by maximizing profits rather than the health of people, fostering trivial medical procedures for cosmetic purposes alone, or contributing to a questionable agenda for eugenics). The use of cadaver fetal cells is also acceptable if the abortion that provides them is spontaneous, not elected. But human life—even potential human personal life—from zygote to a life beyond this world, remains sacred; what ultimately ends in God ought to be inviolable among humans.

Among the Protestant denominations in the United States, some oppose and some support embryonic stem cell research. Many churches do not yet have an official policy on this issue, although their representatives indicate a kind of majority view. Southern Baptists are, as a group, generally opposed to this research, although there is some openness to the compromise position of using cell lines developed from cells harvested in the past. Some leaders among United Methodists, without an official church position, nonetheless asked President George W. Bush to oppose federal funding for embryonic stem cell research. Presbyterians in General Assembly articulated their support of research on cells extracted from embryos that would otherwise be discarded, and affirmed the use of federal funds to make this possible. Acknowledging the significance of concepts like "potential personhood," and the need to "respect" embryos, they nonetheless determined that, whatever form this respect takes, it ought not to have priority over alleviating the suffering of actual people. Members of the United Church of Christ tend to approve embryonic stem cell research, as long as it is motivated by a clear and attainable healing benefit.

Ethicists from a variety of Protestant backgrounds (although seldom speaking for their denominations) also vary in their approaches to stem cell research. Ronald Cole-Turner agrees with others in the United Church of Christ, by approving the extraction of stem cells from embryos, but sets conditions for research in terms of justice issues. Ted Peters of the Pacific Lutheran School of Theology comes down on the side of supporting embryonic stem cell research, because he believes that, on balance, concern for the human dignity not of embryos but of future real persons is more compelling. Gene Outka and Gilbert Meilaender, both Lutherans, share certain theological and philosophical convictions about the irreducible value of the early embryo. Both want to affirm the continuity of human life between an early embryo and an actualized full person. This means that both will consider the taking of the life of an embryo as a violation of the prohibition against killing. Both also want to distinguish the question of embryo stem cell extraction from the question of abortion (in a variety of ways, but primarily because in the former there is no direct conflict between a woman and a fetus).

Outka, however, finally approves of the use of some embryos for the derivation of stem cells. He does so by introducing two conditions or principles that can exempt one from the absolute force of the prohibition. The first and most distinctive is the "nothing is lost" condition. That is, it is possible to relativize the prohibition against killing when those who are to be killed will die anyway. This is precisely the condition that characterizes the situation of spare (or excess) embryos (from in vitro fertilization procedures) destined to be discarded. The second condition is that "other lives will be saved" through this act of killing. Given the ultimate therapeutic aims of stem cell research, this condition is also present. It is the nothing is lost condition that, in the end, allows Outka both to continue to affirm the status of the embryo as an end, and not a means only, and yet to allow the taking of its life for purposes of research. It becomes, then, the basis of ethical acceptance of the derivation of stem cells from some human embryos, and therefore a carefully-circumscribed, but nonetheless positive approval of embryonic stem cell research.

Meilaender, on the other hand, remains firm (though cautious and reluctant) in the conviction that embryonic stem cell research ought not to go forward, or better, that it is not morally justifiable to use human embryos as the source for stem cells. Referencing three other Protestant theologians (Karl Barth, John Howard Yoder, and Stanley

Hauerwas), Meilaender poses three arguments: (1) people should be considered persons not on the basis of potentiality or actuality, not on the basis of capacities, but simply as members of the human community. To think inclusively about the human species should lead us to honor the dignity of even the weakest of living human beings—the embryo—and thus be able to appreciate the mystery of humanity and the mystery of our own individuality. (2) To opt immediately for embryonic stem cell research is to prevent us from finding better solutions to the medical problems this research is designed to address. Given the ethical compromises involved in following this "handy" route, it would be better to "deny ourselves" this remedy, and look to ones that do not denigrate the dignity of people however and wherever they live. (3) The church should bear witness to its beliefs by refusing to make sophistic distinctions, such as those between funding embryonic stem cell research and funding the "procurement" of these cells (the actual extraction of cells from embryos). Meilaender is skeptical that only excess embryos or available cell lines will be used.

Along with evangelical Protestants, Roman Catholic voices have been prominent in the debates surrounding embryonic stem cell research. The position most frequently articulated in the public forum is that of opposition to the derivation of stem cells from embryos. This is the position of the Catholic hierarchy, promulgated through both Vatican and national episcopal channels. It is a position ably supported theologically by spokespersons such as Richard Doerflinger. Yet it is not the only position espoused by Roman Catholic ethicists and moral theologians, or by all Roman Catholics. An alternate position held by many Catholic theologians is basically the "14 day" position, or the theory that an embryo in the first 14 days, prior to implantation in the uterus, is not yet even a "potential" person, and hence need not be protected in the same way as human persons (or even the same as fetuses, considered potential persons after implantation). Neither of these positions goes the route of declaring absolutely that the early embryo is a person, or the route of approving the exploitation of fetuses in utero in later stages of development. Since the debate between these two positions reflects similar philosophical and theological debates in other religious traditions and in the public forum, it is worth looking at it in closer detail.

ROMAN CATHOLIC CONTRIBUTIONS AND THE 14 DAY THEORY

Before turning to the technicalities of the 14 day theory, it should be noted that Roman Catholics (including the official leaders of the church) tend to worry, like people in other traditions, about issues of justice, ecology, and the well-being of the whole earth. Apart from the moral status of the embryo, Catholic concerns are focused on questions of equity in the shared lives of persons across the world. For all the pressures to go forward with embryonic and fetal tissue stem cell research, there is little assurance anywhere that the benefits of this research will be shared in the human community among the poor as well as the wealthy, across racial and gender lines, and in countries of the world's south as well as north. Moreover, as the research goes forward (for it surely will), some safeguards will need to be in place to keep the goals of the research focused on the healing of human persons, rather than on the commodification of human bodies, their tissues and cells. And the specter of human genetic engineering for enhancement purposes (and not only the treatment of diseases and injuries) is never far from the horizon. The other side of tremendous positive advances of modern technology is not to be ignored, not even if our present anxieties lead us desperately to seek means to lift the burdens of one generation or one group of people.

It is important to take preliminary note, also, of the shared community of discourse among Roman Catholic scholars, church leaders, and people. No matter how divided they may be among themselves—regarding the moral status of the early embryo or other particular moral questions—common moral convictions are nonetheless expressed in common language. The Catholic tradition is undivided in its affirmation of the goodness of creation, the role of human persons as agents in cooperation with ongoing divine creative activity, the importance of not only the individual, but also the community, the responsibilities of human persons to promote the health and wellbeing of one another. At the same time, Catholics do disagree profoundly; a key example of disagreement is the debate about the status of the embryo, and the moral evil or goodness of extracting stem cells from it in a way that leads to its demise.

The debate within the Catholic community regarding stem cell research incorporates opposition over something like the 14 day theory, because the Roman Catholic moral tradition has consistently embraced a form of moral realism. At the heart of this tradition is the conviction that creation is itself revelatory. That is, embedded in an ultimately intelligible (though only partially so to humans) created reality is the possibility of perceiving and discerning moral claims of respect in response to every entity, especially human persons. This is what is at stake in the Catholic tradition of natural law. Although there are historical inconsistencies, and even contradictions, in this long tradition, for the most part, natural law has not meant that morality can simply and fully be "read off" of nature, not even with the help of the special revelation of the Bible (although this is a help). What natural law theory does is tell persons where to look—that is, to the concrete reality of the world around us, to the basic needs and possibilities of human persons, and to the world as a whole. "Looking" involves discerning, deliberating, structuring insights, interpretating meanings, taking account of not only what is

similar among entities, but also what is particular in their histories, contexts, and relationships.

Roman Catholic thinkers engaged in discernment about human embryonic stem cell research tend, therefore, to "look" to the reality of stem cells and their sources, i.e., human embryos and aborted fetuses, as well as adult tissues. All answers are not in the Bible, official church teachings, or individual experiences of the sacred. Discernment incorporates all of these sources of insight, but it also requires the knowledge available from the sciences, philosophy, and whatever other secular disciplines provide some access to the reality that is being studied. The ongoing intensity of the debate about the status of embryos bears witness to the notion that not all inquiries can be settled by one discipline, or one interpreter, or within one epistemological perspective.

The argument against procuring stem cells from human embryos is primarily that it entails the death of the embryos. No one disputes this fact, but disagreement rages as to whether this death can be justified. Those who oppose its justification argue that for each human person there is a biological and ontological continuum from the single-cell stage to birth, to whatever one has of childhood and adult life, and then to death. Since a new and complete genetic code is present after fertilization, there is already a unique individual human person, potential in an important sense but concretely and really (inherently) already directed to full actualization. The zygote itself, as a living organism, is self-organizing. The moral status of the early embryo is, therefore, that of a human person; it does not achieve this status by degrees or at some arbitrary point in development; nor is this status simply bestowed upon it by social recognition or convention.

But, if the embryo has the status of a human person, killing it even in order to treat the illnesses of other people cannot—from this Catholic point of view—be justified. No human person, not even an inchoate one like an embryo, can be reduced to a pure means in relation to other ends. Creating or using embryos for research purposes is wrong, because it treats this distinct human being as a disposable instrument for the benefit of someone else. Those who oppose the extraction of stem cells from embryos on the grounds that it involves the destruction of a potential human person are not unsympathetic to the suffering that may be alleviated ultimately through research on stem cells. Part of the argument against the use of embryonic sources for stem cells rests on the identification of alternative sources (particularly adult cells).

On the other side of this debate are Catholic moral theologians, who do not consider the human embryo in its earliest stages (prior to the primitive streak or to implantation) to be a human person, potential or actual. They hold the same meaning for "potency" as do their opponents—that is, these theologians hold, too, an Aristotelian and Thomistic meaning, signifying not an extrinsic or "sheer possibility" potential, but rather an intrinsic principle, already as such actual within a being, directing it to individualized species-specific development (even though the being may not yet be developmentally fully actualized). These moral theologians argue, then, precisely against the view that the early embryo has this inherent potency to become a person. Their argument harks back to a centuries-old Catholic position that a certain degree of biological development is necessary in order for the human spirit (or soul) to be embodied. The conceptus, in other words, must have a baseline organization before it can embody a human person; it must embody the potentiality as well as actuality that will lead to the formation of a human person. Although previous theories of embryological development were grounded in minimal (and to a great extent, erroneous) scientific evidence, proponents of embryonic stem cell research find in contemporary embryology insights that tend to support this developmental theory, rather than defeat it.

Australian Catholic moral theologian Norman M. Ford has presented perhaps the most detailed argument in support of the view that an early embryo is not a potential human person. The plausibility of "delayed personhood" is based on scientific evidence that suggests that the embryo prior to implantation is not yet a self-organizing organism. Fertilization itself does not take place in a "moment," as is sometimes rhetorically claimed; it comes about through a process that takes approximately 24 hours, finally issuing in the one-celled zygote. When the zygote then divides, there comes to be not a definitive, singular organism, but a collection of cells, each with a complete genetic code. In other words, the first two cells, even if they interact, appear to be distinct cells, not a two-cell ontological individual. The same is true as division continues to four-, eight-, and sixteen-cells, each with its own life cycle and nutrients for sustaining its life. Cells multiply and differentiate as they are gradually specified in their potential. Some cells form membranes that will finally enclose an organized individual entity, approximately 14 days after fertilization. It is only then that a unified being can be said to be "self-organizing." Until this time, cells are "totipotential," in that they can become any part of what may ultimately be a human being, or even a human being as a whole.

The genotype of the zygote does not have a built-in blueprint for development, as if it were a miniature person. Only with the formation of a single primitive streak (after implantation) does the totipotency of the embryo actually become a potency to develop into one human person. As Ford puts it, fertilization is not the beginning of the development of the human individual but the beginning of the formative process and development into one (or more human individuals). Many who find this argument plausible tend to express it more simply: since at its early stages an embryo can twin and recombine, there is not here an individualized human entity with the inherent settled potential to become a human person. Critics of the position respond that if an

embryo does not twin or recombine, then it may be supposed that an individual already exists. Ford's analysis goes further than this, however, suggesting that the very undifferentiation of cells prior to implantation disallows the identification of individuality, whether or not there is twinning and recombining.

Since this articulation of the 14 day theory is dependent on the current state of mammalian embryological studies, Catholic moral theologians on both sides of the debate take any new scientific information seriously regarding just what is happening in the first stages of embryological development. For example, studies of the role of first cleavage (of cells) in the development of mouse embryos suggest that an organizational process is already underway. Those who argue that the identity of the embryo is determined right after fertilization use this information to demonstrate a unitary process of self-organization. Those who support the 14 day theory do not deny that something is happening in the early stages of cell cleavage; they maintain, however, that the level of complexity and of organization is as yet too low to settle the individuality of a developing potential person. The embryo in its first stages must develop, in order to be able to implant; only when it reaches this stage is its singularity and individuality settled.

In addition to the argument from lack of individuation, some theologians have noted that the high rate of spontaneous early embryo loss undermines the credibility of the claim that human individuals begin at fertilization. In unassisted human reproduction, the development and loss of early embryos is estimated to be as high as 50–80%. From a Catholic theological point of view, it seems unbelievable that more than half of the individual human persons created by God should populate heaven without ever having seen the light of day in this world. This is a speculative observation, however, and it is generally not used today by those who favor embryonic stem cell research.

There are, then, two opposing cases articulated within the Roman Catholic tradition. This need not leave Catholics (or others) with a kind of "draw." Rather, it opens and sustains a significant conversation that takes account of scientific discoveries, as well as of a larger set of theological and ethical insights. Moral theologians and ethicists attempt to provide reasons for their views that will be open to the scrutiny of all. Both sides claim a certain amount of epistemic humility, since there are only degrees of certainty available regarding the ontological status of the early embryo. Indeed, even official church documents acknowledge that there is no definitive answer to the question of when human individual life begins. In 1974, the "Declaration on Procured Abortion" stated in a footnote that the question of the moment when the spiritual soul is infused is an open question. It acknowledged that there is not a unanimous tradition on this point, and authors are as yet in disagreement. In 1987, an instruction on "The Gift of Life" admitted that its authors were "aware of" current debates concerning the beginning of human life, the individuality of the human being, and the identity of the human person. Pope John Paul II made no decision on the question of delayed ensoulment (or "hominization") in his 1995 encyclical letter, "Evangelium Vitae." And the US Catholic bishops, even in statements made in 2008, have not attempted to settle this question.

Uncertainty is dealt with in different ways by those who oppose and those who support embryonic stem cell research. Those who oppose it argue that even if the presence of the "personal soul" in an embryo is only "probable," this suffices to prohibit risking the killing of a human person. In other words, probability (if not certitude) warrants the safer course. Hence, the embryo from fertilization on is to be treated as a human person, with the kind of unconditional respect due to all members of the human community. This argument is reinforced by a concern for a "slippery slope" along which human persons will cease to be respected, no matter what their actuality or their needs.

For those who conclude that embryonic stem cell research can be permitted, uncertainty works the other way. First, the probability of an early embryo's actually representing an individualized human being is much lower than is argued by opponents of embryonic stem cell research. Although there is no absolute certitude to be gained at this point in time from scientific evidence, the weight of the evidence against there being an individualized human being (incarnated in an embryo prior to implantation) is greater and more persuasive than the evidence for it. Moreover, the low level of uncertainty (for those who hold this position), when placed in relation to the prospect of great benefits for human healing (and some assurance that these benefits are not promised unrealistically) makes it possible—without yielding to a full-blown utilitarianism—to justify using early embryos as sources for stem cells for research. To prohibit all such research on the basis of a low probability of fact, or on a theological stipulation of moral status in the face of questionable accuracy of appraisal, seems itself not ethically justifiable.

Neither side in this debate wants to sacrifice the tradition's commitments to respect the dignity inherent in human life, promote human well-being, and honor the sacred in created realities. When a move forward is advocated for embryonic stem cell research, it need not soften the tradition's concerns to oppose the commercialization of human life and to promote distributive justice in the provision of medical care. The ongoing Roman Catholic conversation on all of these matters can be of assistance to others in a pluralistic society, as long as it remains open to wider dialogue, respectful of all dialogue partners, while retaining its own integrity. This is probably a lesson for all religious and secular traditions.

KEY WORDS

Moral realism Theory of knowledge that affirms the possibility of at least partial corresponence between what is in reality and what is in the mind.

Ontological Having to do with the structure of being.

Primitive streak The initial band of cells from which the individuated embryo begins to develop; it is present at approximately 14 days after fertilization.

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Chapter 68

Stem Cell-based Therapies: Food and Drug Administration Product and Pre-Clinical Regulatory Considerations

Donald W. Fink and Steven R. Bauer

ABSTRACT

The expectation that cellular biologic therapies either consisting of or derived from embryonic, fetal or adult stem cells (stem cell-based products) will provide effective treatments for a variety of current unmet medical requiring replacement, restoration, repair, or regeneration of damaged or diseased tissues and organ systems continues to drive expanding research efforts. Previously limited primarily to the domain of stem cells isolated from bone marrow or peripheral blood used for reconstitution of the hematopoietic and lymphopoietic systems following myeloablative and immunosuppressive treatments, the phenotypes, anatomic sources and potential clinical indications for stem-cell based products has expanded considerably in recent years. Beyond the promise of direct therapeutic efficacy achieved through seeding and repopulating or promulgation of bystander effects that provide trophic support of endogenous cells in areas ravaged by trauma, stem cells are projected to serve as biologic vehicles that may be engineered to deliver functional genes and gene products to discrete target sites where degenerative damage and disease is the consequence of genetic anomalies. Moreover, there is growing recognition that tissue-specific *in vitro* differentiation of stem cells could result in generation of important new model systems suited for investigating the underlying biological basis of disease as well as provide critical testing paradigms to screen potential lead candidate pharmaceuticals for toxicological safety and putative therapeutic efficacy. The scientific underpinnings necessary to support initiation of clinical safety trials in humans involving stem cell-based products have been buttressed by important research findings generated over the past several years. Sustaining ongoing science-driven efforts to identify and critically assess issues pertaining to stem cell safety is key to achieving the ultimate goal of producing novel cellular products that are both safe and effective. The challenge of drawing upon the spectrum of scientific evidence available to assess and guage the safety of biologics produced from stem cells is the responsibility of the Center for Biologics Evaluation and Research (CBER) within the Food and Drug Administration (FDA). Detailed information pertaining to the safety and efficacy of investigational stem cell-based products is reviewed by staff within the Office of Cellular, Tissue, and Gene Therapies (OCTGT), CBER. The stated mission of CBER is to ensure the safety, purity, identity, potency and efficacy of new biological products for the prevention, diagnosis, and treatment of human diseases, conditions, or injury. Mission objectives are achieved, in significant measure, using a regulatory review process that is founded squarely on scientific principles thus making available to the public innovative new treatments in as timely a manner as is feasible. This chapter describes the approach used by CBER to evaluate investigational stem cell-based products.

INTRODUCTION

Stem cell-based products are cellular constructs consisting of, or derived from, populations of stem cell progenitors. They represent examples of complex and dynamic biological entities. Stem cells are characterized by their capacity for expansion, through self-renewing proliferation, and their the capacity for differentiation into a variety of cell types, limited primarily by constraints imposed by the phenotype lineage-forming potential of the stem cell progenitors. Once transplanted into patients, stem cell based preparations are expected to interact intimately with, and be influenced by, the physiological milieu of the recipient. Elucidation of the molecular mechanisms that control stem cell self-renewal and commitment to any of a variety of cell fates pose vexing challenges to the field of developmental biology, and it is the responsibility of the Food and Drug Administration (FDA) to confront these complexities, as the agency executes regulatory oversight of investigational stem cell-based products. Numerous fundamental questions pertaining to regulation of gene expression, cell–cell interaction, and control of cellular differentiation remain under active investigation within the scientific community. The science-based regulatory review approach implemented by Center for Biologics Evaluation and Research, Office of Cellular, Tissue and Gene Therapies (CBER/OCTGT) for evaluation

of novel stem cell-based products is consistent with practices in evidence throughout the FDA. This regulatory approach is designed to facilitate progress in the development of new biological treatments, ensuring to the greatest extent possible the safety of patients enrolled in investigational clinical studies conducted to support potential future licensure of these innovative biological products.

RESOURCES TO DEVELOP RECOMMENDATIONS FOR THE MANUFACTURE AND CHARACTERIZATION OF STEM CELL-BASED PRODUCTS

Irrespective of their source, constructs derived from stem cells are categorized as somatic cellular products. The legal authority for FDA to regulate stem cell-based biological products is derived from two Congressional Acts, the Federal Food, Drug and Cosmetic Act (FD&C Act), and the Public Health Service Act (PHS Act). Stem cell-based somatic cellular products are regulated as Biologics, under Section 351 of the Public Health Service Act. To initiate first-in-human clinical studies, designed to generate information pertaining to the safety and possible effectiveness of a previously-untested stem cell-based product intended for the treatment, mitigation, or cure of disease, an Investigational New Drug application (IND) must be compiled and submitted to CBER/FDA for review and assessment. An IND is a request for permission to be granted by the FDA to proceed with patient administration of an investigational stem cell-based product. Among the essential items included in the IND submission document is detailed information that describes the manufacture and characterization of the investigational stem cell-based therapy, along with a compilation of results obtained from preclinical studies conducted in animals, and a well-designed, prospectively written, adequately controlled clinical protocol. Due to the inherent complexity of stem cell-based products, coupled with continuous accumulation of research-generated information about their characteristic biological properties, individual investigators and corporate sponsors, alike, are encouraged to request a pre-meeting with CBER/FDA before submitting a completed IND application for evaluation. Within the context of a pre-IND meeting, sponsors are able to receive input from CBER/FDA staff that represents current thinking with respect to regulatory expectations that will be addressed by the information provided in an IND application. FDA recommendations and requirements regarding characterization of stem cell-based products are derived from a variety of sources, including product-specific regulations stipulated in the Code of Federal Regulations (CFR), relevant guidance documents, input received from select FDA advisory committees, and direct interactions with the scientific community.

Code of Federal Regulations (CFRs)

The CFRs represent the structural backbone of the FDA's regulatory requirements. Regulations encode details of the laws enacted by Congress, and serve as the blueprint for their implementation. Regulations applicable to the clinical investigation of unproven stem cell-based products include those outlining requirements that govern submission of an investigational new drug application (21 CFR 312), and compliance with current good manufacturing practices (21 CFR 210 & 211). These regulations were drafted by the Agency, and approved by the Executive Branch. During early clinical development, the primary objective of this specific set of regulations is to ensure, to the greatest extent possible, that patients enrolled in clinical trials receive investigational stem cell-based products with an acceptable safety profile, taking into account the balance between identified risk signals, clinical indication, and possible patient benefit. Ultimately, the goal of performing clinical trials is to collect sufficient safety and efficacy data on the investigational product, in accordance with regulatory requirements, to permit license of the product, which allows for widespread interstate commercial distribution to patients.

Relevant Guidance Documents

Regulations outlined in the CFRs that are germane to the submission of an IND may be applied broadly to a diversity of therapeutic products. In contrast, guidance documents are often developed for the purpose of reflecting the FDAs current thinking and expectations concerning the development of specific product classes. Guidance documents written and approved in accordance with intra-organization procedures represent the Agency's interpretation of published regulations. In addition to its own guidance development efforts, the FDA interacts with outside regulatory organizations participating in wider global initiatives, such as the International Conference on Harmonization (ICH), resulting in development of instructive documents that pertain to the manufacture, preclinical and clinical testing of many types of biological products. The principles outlined in a number of these guidance documents have been developed collaboratively, and are applicable to the evaluation of stem cell-based products.

Scientific Interactions

To facilitate the development of guidance documents and regulatory review approaches that are pertinent to the development of stem cell-based biological products, CBER/OCTGT review staff participate in regularly-scheduled internal meetings, to discuss relevant scientific issues pertaining to specific biological products, and to promote consistency in their regulation. In addition, the OCTGT staff is involved in various outreach activities, including regulatory

and scientific society meetings where interested parties are afforded the opportunity to interact directly with individuals involved in the regulation of stem cell-based products. FDA staff and members of the regulated community benefit mutually from interactions that occur outside of the formal regulatory process. These exchanges allow for an enhanced understanding of, and greater appreciation for, each other's perspectives. A number of CBER/OCTGT review scientists also maintain active laboratory-based research programs in cellular or developmental biology, as well as in other areas relevant to the regulation of stem cell-based products. This strategic staffing approach, designated the research-reviewer model, is critical to the FDAs effort to sustain state-of-the-art scientific expertise and credibility, as it conducts the complex task of regulating stem cells and developing regulatory policy that affects the stem cell therapy field.

The practical experiences of academic clinical institutions and corporate entities involved in the development of stem cell products play a key role in shaping FDA recommendations pertaining to the regulation of stem cell-based products. The communication of these broader experiences through meetings with CBER/OCTGT staff, and presentations made at scientific society meetings, or in front of CBER's Cellular, Tissue and Gene Therapy Advisory Committee (CTGTAC) is vital to informing the regulatory process. Within the context of a CTGTAC meeting, the FDA is able to obtain advice in a public forum on scientific issues that may affect its approach to the regulation of stem cell-based products. The contributions of the CTGTAC to execution of the CBERs regulatory oversight of stem cell-based products are not to be underestimated or overlooked. Two notable meetings of the CTGTAC, one conducted in July of 2000 convened to consider use of stem cell-derived products for treatment of neurological disorders, and the second held in April of 2008 focused on safety assessment of products derived from human embryonic stem cells, represent examples of the CTGTAC's contribution to the CBERs regulation of stem cell-derived products. Transcripts of these meetings are available through the CBERs public website (http://www.fda.gov/cber/advisory/advisory.html).

Another important example of scientific resource leveraging, that facilitates development of recommendations pertaining to the regulation of stem cell-based products, is collegial interagency interaction between the FDA and the National Institutes of Health (NIH), under the umbrella of formalized Memoranda of Understanding agreements (MOUs) that allow for information exchange on the subject of translational research. The FDA benefits from the scientific expertise provided by the NIH extramural program managers and intramural research scientists, while the NIH derives benefit from the FDAs advice related to development of important NIH grant funding initiatives designed to expedite translation of scientific research from bench to bedside. These MOU agreements serve the FDA-regulated community, the NIH-funded scientific research community, and the public by focusing critical resources on the development of novel technologies, such as stem cell-based products, to address challenges posed to the public health in the form of significant unmet medical need.

In summary, the FDA receives input from a variety of sources that facilitates formulation of recommendations germane to the evaluation of stem cell-based products for evidence of their safety and effectiveness. Guidance documents are developed that contain recommendations encompassing the Agency's current thinking with respect to the regulation of cellular products, including those composed of stem cells. These documents are written to accommodate updating, as appropriate, in order to reflect continuing scientific advances, and the accumulation of regulatory experience. When making specific decisions pertaining to investigational new drug applications for stem cell-based products, the FDA takes into account evidence of risks posed versus any possible benefit that may be realized for each stem cell-based product being evaluated within the context of an adequately designed clinical trial.

CRITICAL ELEMENTS FOR DEVELOPING A SAFE STEM CELL-BASED PRODUCT

The current, comprehensive approach the CBER uses to evaluate investigational stem cell-based products is depicted schematically in Figure 68-1. Highlighted are essential elements sponsors need to address in order for CBER/OCTGT review staff to perform thorough safety assessment of a stem cell-based product, prior to authorizing permission for initiation of an early phase clinical safety trial. The multifaceted review process encompasses the primary objective of assuring the safety of stem cell-based cellular products. Beginning with "Source Controls," each individual step involved in production of an investigational stem cell-based cellular product intended for clinical testing is subject to careful scrutiny. At the core, evaluating the safety of a stem cell-based cellular product is about establishing and maintaining control over every facet of the process developed to produce the product designated for clinical study. It is only in this manner that the degree of safety is maximized, and consistency from one cell preparation to the next assured.

The individual elements of this comprehensive review approach are not to be viewed as isolated one from the other, but rather as representative of interconnected aspects that constitute the review framework. Acquisition of the starting biological materials obtained from eligible consented donors; derivation, expansion, manipulation, and characterization of established stem cell lines; generation of differentiated cellular preparations; and preclinical proof-of-concept/toxicological testing conducted in appropriate animal models are all items considered in an interwoven review process. An example of the nature of this review approach is demonstrated in the principle of traceability, or linkage. In those situations when an unanticipated

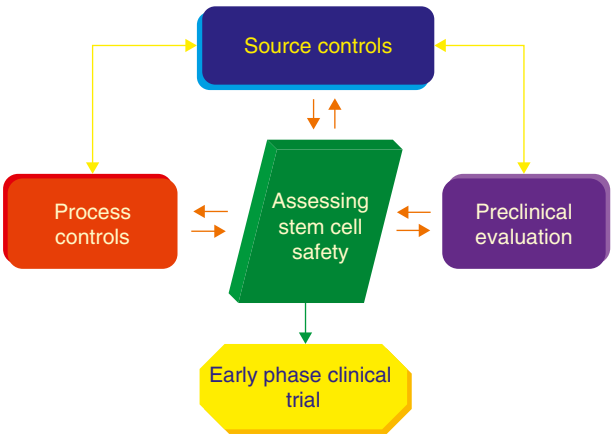


FIGURE 68-1 Representation of elements deemed necessary for assessing the safety of novel human embryonic stem cell-derived cellular products.

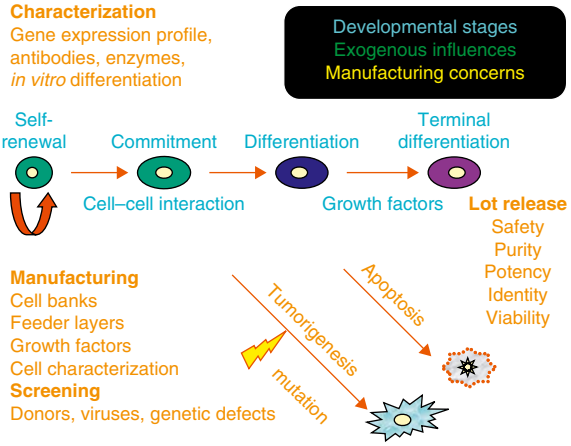


FIGURE 68-2 A hypothetical cellular differentiation pathway is used to illustrate the complexity of regulatory oversight of stem cell products. Mechanisms that control biological processes, such as self-renewal or lineage commitment, include changes in gene and protein expression that lead to differentiation (steps in development, blue text), and interactions with the host through microenvironment cell–cell interactions or growth factors (influences on development, green text). Regulatory concerns associated with these processes during *in vitro* stem cell manufacture are shown (yellow text).

adverse event occurs during the course of an investigational clinical trial, traceability allows investigators and study reviewers alike the opportunity to link back from the patient through the stem cell preparation process, all the way to the initial acquisition of biological materials used to generate the initial starting material. Traceability permits evaluation of an unexpected adverse event in the context of clinical, manufacturing, and preclinical testing issues, allowing informed decisions to be made that assist in ensuring the status of patient safety. Finally, to increase the degree of safety assurance attainable through product characterization, it is essential to develop analytical methods that are sufficiently sensitive for the detection of cells within a preparation that exhibits undesirable characteristics. To achieve this end, it is necessary to identify characteristic cellular markers that function as reliable indices of product safety. Accordingly, during the development phase that precedes initiation of a first-in-human clinical study, efforts are undertaken to identify product characteristics associated with positive outcomes, and that could be capable of predicting clinical effectiveness, as well as establishing those product features that anticipate the potential for adverse events to occur.

Manufacturing and Characterization Issues Pertaining to Stem Cell Products

Ensuring the safety of patients receiving an investigational therapeutic product not tested previously in humans is a primary objective of the FDA's oversight of stem cell-based products. Attention focused on the details of product manufacturing and characterization is essential to safety assurance. The information provided in this section describes current CBER/OCTGT reviewer recommendations intended to ensure that investigational stem cell products administered to patients during the course of a clinical trial meet agency expectations in terms of safety, purity, identity, and potency. Figure 68-2 provides illustration of the complexities inherent in stem cell biology, and depicts challenges associated with development of stem cell-based products.

Control of Source Materials is the First Step in Safety Assurance

Providing demonstration of an acceptable safety profile for an investigational, stem cell-based product prior to initiating an investigational clinical study begins at the stage of determining tissue donor eligibility. For cellular products derived from human embryonic stem (hES) cells, this encompasses screening and testing of gamete donors providing the eggs and sperm used to generate embryos from which embryonic stem cells are isolated. A description of the specific sources from which stem cells are acquired includes information about the tissue of origin and cell type, such as hematopoietic, neuronal, or embryonic stem cells, as well as details indicating whether donor cells are obtained following induced mobilization from an anatomical location that is difficult to access.

1. Determining Donor Eligibility

One of the FDA's principal public health obligations is to prevent the introduction, transmission, and spread of communicable diseases. Biological therapies comprised of stem cells pose a risk for transmission of communicable disease from donor to recipient. In the FDA's final rule

on determining the eligibility of human cells, tissues, and cellular and tissue-based products donors, acceptance of tissues obtained from allogeneic (non-self) donors as a suitable source material for producing stem cell-based products depends on the screening and appropriate testing of donors for a panel of relevant communicable diseases. This includes testing for human immunodeficiency virus types 1 and 2, hepatitis B virus (surface and core antigen), hepatitis C virus, and *Treponema pallidum* (syphilis). In addition, for donors of leukocyte-rich cells of tissues testing for human T-lymphotropic virus types I and II, and cytomegalovirus (CMV) is required. Testing is performed by qualified laboratories, using FDA-licensed, approved, or cleared test kits. Within the autologous setting, where patients serve as their own source of stem cells, testing for infectious disease or adventitious agents is relevant when *ex vivo* culture methods are used during processing which could promote propagation of an adventitious agent within a given stem cell product, or increase the risk for cross-contamination of other biological products manufactured within the same facility. An additional level of safety is provided by required labeling of cellular and tissue products. In clinical settings when the products are intended for autologous use, they should be labeled "FOR AUTOLOGOUS USE ONLY." Circumstances may arise when it is not possible to either conduct or complete screening and testing necessary for determining donor eligibility. In this case, cellular and tissue products should bear the label "NOT EVALUATED FOR INFECTIOUS SUBSTANCES."

In addition to testing for evidence of infectious diseases, IND applications to the Agency should include a description of other serologic, diagnostic, and clinical history information obtained for the donor. It may be important to conduct further donor characterizations, such as typing for genetic polymorphisms, or assessment of major histocompatibility complex (MHC) loci that may play a role in determining whether transplanted stem cell products will engraft successfully, given that engraftment failure may occur due to donor–host immunological incompatibility.

Donor eligibility determination is required for non-sexually intimate gamete donors providing unfertilized eggs or sperm to be used specifically for generation of human embryonic stem cells (hES) isolated from the inner cell mass of fresh 5–7-day-old blastocysts. When the collection of gametes is not for the expressed purpose of reproductive use, volunteer gamete donors are to be screened for high-risk behaviors, and tested for evidence of infectious diseases, using FDA-licensed, approved, or cleared test kits. On the occasions when extra, unused, cryopreserved fertilized embryos obtained from a fertility clinic serve as source material for derivation of the human embryonic stem cell lines, donor eligibility determination is not required for embryos created and stored prior to 25 May 2005. The requirement for gamete donor screening and testing is complicated by the fact that sexually intimate partners providing egg and sperm initially intended for personal reproductive use are exempted from donor eligibility testing requirements. In these situations, gamete donors may not be available for relevant communicable disease screening and testing. Under these circumstances, once cultures of derivative undifferentiated hES cells have expanded to numbers sufficient to support adequate sampling, the cells may be assessed directly for evidence of infectious diseases using PCR-based tests qualified and validated for this purpose. Direct testing of derivative cell lines for relevant communicable diseases is also performed for human embryonic stem cell lines isolated from embryos created and cryopreserved prior to 25 May 2005. Archiving of gamete donor-derived blood samples permits screening for additional disease markers as new information and diagnostic techniques become available, providing an added measure of safety assurance.

In addition to screening for infectious diseases, a role for molecular genetic testing has been contemplated as a component of an overall assessment program to establish the suitability of particular hES cell preparations for use in developing a stem cell-based product. Information obtained from this type of evaluation could serve to determine whether a preparation of hES cells represents the best-qualified source material for generating a cellular product, in the context of a particular disease setting. As a hypothetical example, genetic testing could detect, in theory, mutations in specific target genes, such as α-synuclein, that have been linked to autosomal dominant familial Parkinson's disease. Detection of such genetic anomalies in a population of hES cells could negate their use in generating neuronal progenitor cells for treating a number of neurodegenerative conditions, including Parkinson's disease. Presently, a relatively small number of genes are known to be directly responsible for causing disease in a monogenetic fashion, or to operate as suspected contributors to aberrant physiological function. Undoubtedly, this number will expand, due to advances in techniques for identifying, isolating, and analyzing genes, coupled with an increasing abundance of information certain to become available as one outcome of the human genome sequencing projects. In addition, it is expected that a great deal more will be learned as to how multiple gene products, each contributing incrementally to an overall outcome, will predispose individuals to development of certain diseases. Barring significant improvements to existing technology, it is impractical to expect screening of every donor or hES cell preparation for the entire panoply of disease-associated genes. In this context, molecular genetic testing would be conducted only for targeted genes that are relevant to a specific clinical indication. It is important to note that, at present, molecular genetic testing is not required by the FDA as part of the approach used to evaluate the safety of

an investigational stem cell-based therapy intended for use in a clinical trial.

2. Manufacturing Components and Procedures

The approach adopted by CBER/OCTGT for regulation of biological products, including stem cell-based products, involves scrutiny of the manufacturing process used to produce the stem cell-based product, and analysis of final product testing procedures. Often the production of a stem cell-based product requires a multi-step process that must be performed using aseptic techniques, as terminal sterilization of the final product is not feasible. An error in the execution of virtually any manufacturing process step could affect the safety, purity, and potency of the stem cell product, along with allowing for the introduction of undesired adventitious agents. CBER/OCTGT review staff evaluate all procedures used during the production, purification, and harvest of a cellular therapy product. Details describing methods used for cell collection, processing, and culturing conditions, including mechanical or enzymatic dispersion, cell selection methods, and a description of the optimized cell culture system, are important. In addition, CBER/OCTGT review staff examine details pertaining to the quality of all reagents used in the manufacturing process. Examples include reagents that are required for promoting cellular growth, eliciting differentiation, and selection of target cell populations, specifically, bovine serum, trypsin, peptide growth factors, cytokines, monoclonal antibodies, antibiotics, cell selection devices, and specified culture media. Use of FDA-approved reagents, when available, is encouraged, however, when this is not possible, additional testing of a reagent may be necessary to ensure its safety and purity. Add-on testing could include assessment of sterility, endotoxin levels, mycoplasma contamination, and evaluation for the presence of adventitious agents. Analysis of reagent purity, as well as additional analytical tests designed to demonstrate the absence of potentially harmful substances, might be indicated.

One issue that receives considerable attention is the inclusion of bovine-sourced serum or other critical components as media supplements. Of prominent concern is the potential risk for contamination by prions, the causative agent of bovine spongiform encephalopathy (BSE). Several options are available to safeguard against inadvertent transmission of BSE to patients through transplantation of contaminated stem cell-based products. First, cell cultures may be maintained in medium supplemented with bovine serum, once it is demonstrated that the serum is sourced from donor cows reared for the entirety of their lives in herds maintained in countries certified as BSE-free. A second alternative is to replace bovine serum with clinical grade serum sourced from humans. In this case, testing of the serum donors or final pooled serum product for infectious human diseases is essential. The third option is to develop a chemically defined, serum-free medium, thus obviating altogether risks associated with serum supplementation.

Derivation of Stem Cell Lines

1. General Principles

An IND application should contain information about the origin of any stem cells used to produce an investigational therapy, and include details about the source tissue, manner of collection, and methods for cell expansion. Demonstrating rigorous control over standardized practices and procedures used during isolation, derivation, and maintenance of stem cell lines ensures the integrity, uniformity, and reliability of candidate stem cell-based products. When a stem cell therapy is derived from a non-human source, it meets the definition of a xenotransplantation product. As defined in the PHS guideline on this topic, xenotransplantation is any procedure that involves the transplantation, implantation, or infusion into human recipients of either: (1) live cells, tissues, or organs from a nonhuman animal source; or (2) human body fluids, cells, tissues, or organs that have had *ex vivo* contact with live nonhuman animal cells, tissues, or body organs. Guidance documents provided by the agency describe additional testing requirements necessary to address potential risks posed to humans resulting from the use of xenotransplantation products. Details that pertain to animal husbandry practices, herd health surveillance, adventitious agent testing of derivative stem cell products, and archiving of animal and patient specimens are all taken into consideration by the agency in association with the use of xenotransplantation products.

Finally, once a stem cell line is established, a description of prospective testing used to characterize that stem cell line should be provided in an IND application submitted to the FDA/CBER. A representative panel of tests that includes, but is not limited to, the following parameters is recommended: (1) for hES cell lines, demonstration of pluripotency; (2) karyotypic and chromosomal analysis; (3) assessment of proliferative potential and growth characteristics; (4) evaluation for the expression of markers indicative of undifferentiated stem cells versus differentiated derivatives; (5) analysis of the stability of cell lines over time in tissue culture and following prolonged periods of cryopreservation storage. These recommendations also apply to cell lines comprised of inducible pluripotent stem cells (iPS). Inducible pluripotent stem cells are generated by nuclear reprogramming of adult, functionally-differentiated somatic cells, such as skin-derived fibroblasts, and appear to replicate the biological properties of embryonic stem cells. For iPS cell lines created by vector-mediated delivery of transcriptional regulating factors, regulatory requirements governing gene transfer products become a component of the review process.

2. Human Embryonic Stem Cells

The derivation of an hES cell line requires additional considerations be taken into account. Because hES cells represent a complex, dynamic biological entity characterized by

robust proliferative and differentiation potential, failure to standardize procedures for expansion and maintenance of cultured hES cells could result in unintended alterations of their intrinsic properties. For example, the initial cell seeding density, the frequency with which culture medium is replenished, the quality of the reagents used to supplement the culture media, and the degree of confluency cell cultures are permitted to achieve prior to subdividing could all impact cultured hES cell characteristics. Standardization of procedures used to acquire egg and sperm from eligible volunteer donors, perform *in vitro* fertilization, and isolate cleavage-stage embryos is important to the generation of quality, undifferentiated hES cell cultures. When cryopreserved embryos serve as the starting source material for deriving self-renewing hES cell cultures, it is essential that critical information is documented and records maintained regarding the reagents and procedures used to perform embryo cryopreservation. This is the case particularly when more than one infertility clinic is providing cryopreserved embryos as source material for hES cell derivations. Differences in the concentration and type of cryoprotectant used, rate of initial cell freeze down, and routine length of cryopreservation storage may result in variability that precludes consistency from preparation to preparation. Perhaps the most significant issue with respect to use of frozen fertilized embryos is specification of prospective criteria used to ascertain the acceptability of a thawed, cryopreserved embryo as source material for generating hES cell cultures.

Historically, undifferentiated hES cells teased from the inner cell mass of cleavage-stage embryos by mechanical or enzymatic means have been propagated on layers of irradiated, non-replicating feeder cells, frequently comprised of fibroblasts obtained from fetal mice. This is due to the fact that simply adding growth factors and cytokines to the culture media appears insufficient to maintain hES cells in an undifferentiated state, thus the presence of the underlying feeder cells is required. Accordingly, as indicated above, stem cell therapies derived from hES cells cultured on layers of nonhuman cells meet the definition of xenotransplantation products, and are subject to requirements specified in various guidance documents pertaining to the regulation of xenotransplantation products. This circumstance persists even if hES founder cell lines are migrated from nonhuman feeder layers to feeder-free conditions during the process of developing a stem cell-based product. Regardless of the species of origin for feeder layers used to support hES cell cultures, detailed information should be supplied that indicates whether or not feeder cells are derived from primary cell cultures, or established cell lines, describes maintenance of the feeder cells, provides an assessment of feeder line stability (if derived from established cell lines), and includes details regarding the establishment of feeder cell banks (passage number for cells stored as a cell bank, as well as the passage number for cells used during production of the stem cell product).

Histocompatibility constitutes an additional safety issue meriting consideration with respect to the characterization of cell lines established following differentiation of hES cells. Initial reports suggest undifferentiated hES cells derived from the inner cell mass of blastocyst stage embryos exhibit very low levels of major histocompatability complex class I proteins (MHC-I), thus raising the possibility that hES cells might be immune-privileged. This has engendered the hope that products derived from hES cells will be immunologically silent, and thus remain undetected by the recipient's immune surveillance system following transplantation. Moderate increases in MHC-I are noted in conjunction with *in vitro* or *in vivo* differentiation of undifferentiated hES cells. Inflammatory cytokines, generated as part of the inflammatory response, up-regulate MHC expression considerably. In the context of cellular transplantation, induction of an inflammatory response and subsequent up-regulation of MHC-I could enhance the risk for *in vivo* rejection. At this point it is uncertain whether the level of MHC class I expressed in undifferentiated hES cells is sufficient to elicit a vigorous, let alone even a tepid immunologic rejection response, however, it does appear that characterization of MHC profiles for stem cell products derived from hES cells is warranted. Generation of autologous iPS cells is intended to mitigate potential risk posed by development of immunohistoincompatibility associated with a stem cell-based product resulting from the differentiation of allogeneic, undifferentiated hES cells.

Testing of Stem Cell Products: Safety, Identity, Purity, and Potency

A challenge to the evaluation of stem cell-based products is accurate assessment of their safety, identity, purity, and potency. Testing of process intermediates and final stem cell products intended for patient administration is comprehensive and includes, but is not limited to, microbiological testing (sterility, mycoplasma, and adventitious viral agents), as well as assessments of various product parameters, such as unique product identity attributes, purity (freedom from contaminants introduced during the manufacturing process), viability, and potency (biological activity). Appropriate analytical tests selected from this battery of representative tests should be performed at various points throughout the manufacturing process, providing a check on the process itself in order to ensure the quality and reproducible consistency of stem cell products produced over time.

Identity and purity testing may involve biological, biochemical, and/or functional characterization. Tests to demonstrate stem cell product identity and purity should be designed to determine the degree to which characteristics of a manufactured stem cell-based product conform to expected and desired properties. Potency, as defined in the Code of Federal Regulations (21 CFR §600.3[s]), is the "specific ability or

capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." In many circumstances, the mechanism of action for a stem cell-based product is unknown, however, it is expected that the outcome of testing for product potency will correlate with the likelihood to elicit a desired clinical effect. Due to the fact that multipotent stem cells are expected to undergo additional differentiation following patient administration, prior to mediating a measurable clinical effect, the problem of deciding what to evaluate as an index of potency, and how to correlate particular measurements with a stem cell product's final outcome, is a notable challenge. Since direct measurement of the potency of a stem cell-based product may prove daunting, because of a lack of appropriate in vitro or in vivo assays, alternative approaches that assess surrogate biomarkers or activities providing an estimate of potency are considered adequate for initiation of early phase clinical trials. In order to comply with regulations for FDA licensure, development of a valid potency assay that is used for release of the final formulated stem cell-based product is required. It is expected that a suitable potency assay be in place prior to commencing a pivotal phase 3 clinical trial to confirm safety and determine efficacy of the investigational stem cell-based product.

GENERAL EXPECTATIONS FOR CONTROL OF THE MANUFACTURING PROCESS

Control of the Manufacturing Process

A principle that applies broadly to the entire range of complex biologics, including stem cell-based products, is demonstration of control over the manufacturing process used to produce a biological product. In terms of significance, this principle is equivalent to the objectives of thorough product characterization and adequate final product testing for ensuring the safety, identity, purity, and potency of stem cell products. Consequently, strict adherence to standard operating procedures (SOPs) and quality control (QC) of raw materials, reagents, and manufactured product intermediates, in addition to comprehensive final product testing, is essential to ensure that the final formulated stem cell-based product will be acceptable for administration to human subjects. Exerting control over the manufacturing process is crucial for the consistent production of stem cell-based products.

Current Good Manufacturing Practices (cGMPs)

Current Good Manufacturing Practices (cGMPs), as set forth in 21 CFR 210 and 211 of the Code of Federal Regulations, convey principles that apply to stem cell products encompassing both the physical attributes of the facility where products are manufactured, and the processes and procedures specific to the cellular products prepared within a particular manufacturing facility. The following are representative examples of expectations set forth in the cGMPs. Evidence of appropriate written protocols applicable to each stage of product manufacturing and characterization is expected. Standard operating procedures documenting all critical information pertaining to stem cell-based therapy production should be established and adhered to. Quality oversight exercised by the sponsor is important at each stage of product development and involves two elements, QC and quality assurance (QA). Through these mechanisms, individuals assure that manufacturing and characterization testing have been properly performed, that finished product meets specified release criteria, and they maintain independence from and are not directly subordinate to individuals responsible for conducting the specific tests and filing detailed reports. During the final stage of biological product development, namely execution of a pivotal phase 3 clinical study, validation testing of the processes and assays used during product manufacturing and characterization should be performed. The FDA recently issued a final rule that exempts compliance with cGMPs for the manufacture of investigational drugs, including stem cell-based products, used in early stage pilot and phase 1 clinical trials. A guidance for industry on GMPs for phase 1 clinical trials, issued in conjunction with the rule discussed above, describes how manufacturers may implement manufacturing controls to comply with the statutory requirement (Section 501 (a)(2)(B) of the PHS Act) for complying with current good manufacturing practice. It is expected the manufacture of investigational stem cell-based products for early phase clinical testing will be performed in accordance with well-defined written procedures, using adequately-controlled equipment within a suitable manufacturing environment, as documented by accurately and consistently recorded data generated during manufacture and in-process/final product testing.

PRECLINICAL EVALUATION SUPPORTS SAFETY ASSESSMENT: PROOF-OF-CONCEPT AND TOXICITY TESTING IN ANIMAL MODELS

In addition to careful management of the process used to manufacture a stem cell-based product along with intentionally-designed analytical testing used to perform characterization and assure its quality, preclinical evaluation of cellular products consisting of, or derived from, stem cells in appropriate animal models is a key element in the overall safety assessment process. Preclinical testing focuses on two primary objectives: supportive scientific justification for the use of a particular cellular product for a specified disease indication (i.e., demonstrating "proof-of-concept") and assessment of the potential toxicity of the stem cell-based product. Given

the core biological attributes of stem cells, namely a propensity for self-renewal and differentiation potential that could result in outcomes of teratoma and ectopic tissue formation (Figure 68-3), experimental animals should be thoroughly and carefully examined following transplantation of stem cell products, for evidence of unregulated growth, genesis of inappropriate cell types, and formation of ectopic tissues.

Demonstrating Proof-of-Concept

Investigations performed using animal transplant models of human disease constitute an essential component of the assessment paradigm used for judging the safety and rationale for administration of stem cell-based products in humans. Results from these proof-of-concept animal model studies serve to substantiate a rationale for conducting a proposed clinical trial with an investigational stem cell product. Although the ideal animal model should reflect the anatomical features and pathophysiology of the targeted human disease as closely as possible, it is recognized that, unless the disease occurs spontaneously in an animal species, such models will not replicate the clinical situation in an identical manner. Thus, chemical, surgical, and immunological methods are routinely employed to create these models, resulting in damage to neural tissue; induction of diabetes; simulation of myocardial infarction, stroke and hypertension; or compromised organ function. In selected situations when focal monogenetic lesions are known to cause disease, it is possible to create animal models in which the culpable gene is either eliminated, or overexpressed, resulting in the generation of models that recapitulate features of human disease-specific pathologies.

Proof-of-concept demonstration for a stem cell-based product in an animal model of human disease is encouraged for the following reasons:

1. to establish a rationale that supports initiation of clinical trials;
2. to permit concurrent measurement of bioactivity and safety endpoints;
3. to explore the dose-response relationship between a stem cell-based product and an activity/safety outcome; and
4. to facilitate optimization of the route of administration.

Demonstrating that a restorative effect occurs following implantation of an investigational stem cell-based product into an animal model of human disease provides circumstantial evidence for anatomical/functional integration within the host physiology. If corroborated by histopathological data, this proof-of-concept information serves to provide support for hypotheses underlying the mechanism of action of a stem cell-based product. It is important that the clinically relevant route of administration, and intended clinical delivery system are used in the animal models to the extent feasible. Immune tolerance to the implanted investigational human stem cell product, and comparability between cell phenotypes are important considerations when determining whether the use of an analogous stem cell-based product derived from animals is necessary and/or beneficial.

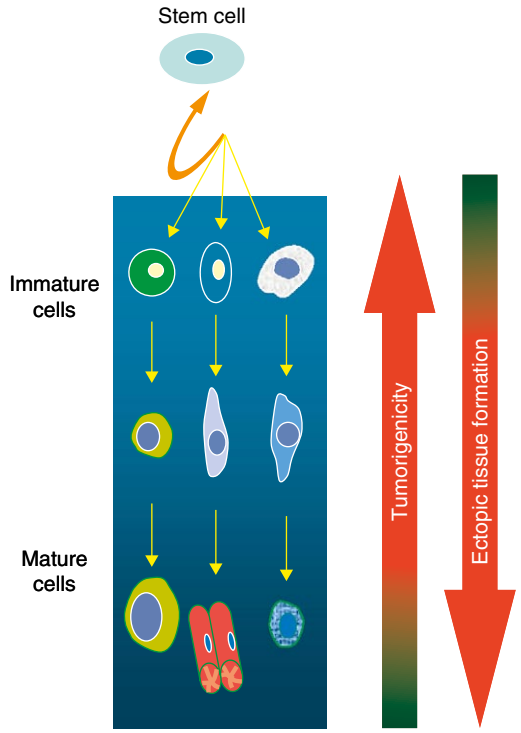


FIGURE 68-3 Schematic representation of undesired outcomes that could occur subsequent to administration of a stem cell-based product, due to the intrinsic proliferative and differentiation properties of stem cells.

Toxicological Assessment

Appropriate evaluation of the safety of an implanted stem cell-based product is an important aspect of the overall development program. Toxicity endpoints are selected that focus on the potential for both local and systemic effects to occur. Depending on the nature of the stem cell-based product being tested, studies conducted in normal animals or animal models of disease should take into consideration assessment of the following, in response to implantation of the stem cell-based product:

1. implant site reaction;
2. any inflammatory response observed in target or non-target tissues;
3. differentiation/ phenotype expression of the stem cell-based product post-implant;
4. the capacity for the cells to survive, migrate and integrate into the host physiology post-implant;
5. observed behavioral changes;
6. morphological alterations in either target or non-target tissues; and
7. evidence of tumorigenicity or ectopic tissue formation.

Completing a comprehensive microscopic evaluation following implantation of a stem cell-based product into animals provides a fundamental assessment of the overall safety of the product intended for administration into humans. The morphological data obtained from such an analysis provides insight into other, in-life, non-terminal evaluations of the animals (i.e., behavioral, imaging modalities neurologic, cardiac, and clinical laboratory changes). Histopathological evaluation can encompass assessment of acute and long-term cell survival, the extent and pattern of cell migration, evidence for differentiation and plasticity, indication of hyperplastic growth or tumorigenicity, and anatomical/functional integration. The extent of interpretable safety information extracted from investigations conducted in animals will be a direct function of the experimental study design, as well as technical limitations associated with the specific test methodologies. For example, for methodologies used to identify and track implanted populations of stem cells, investigators recently reported success related to monitoring stem cell migration *in vivo* following implantation using high resolution *in vivo* magnetic resonance imaging or sensitive methods for detecting visible light emitted from marked stem cells. These types of non-invasive monitoring tools will enable determinations to be made as to whether migration of a stem cell-based product occurs, and if so, whether it is associated with a deleterious outcome.

Tumorigenicity or hyperplastic, unregulated cellular proliferation, and ectopic tissue formation are important safety issues for stem cell-based products, particularly those that are derived from hES cells. Concern as to the ramifications of dysregulated cellular replication following implantation arises from one of the fundamental characteristics of true stem cells, namely their capacity to effect expansion through self-renewing proliferation. For hES cells, concern is heightened, due to their pluripotential character and proclivity for generating malignant teratomas when injected in sufficient numbers into immunodeficient strains of mice. This property, alone, provides a persuasive argument against the administration into humans of stem cell-based products consisting exclusively of unmodified, undifferentiated hES cells. The critical issue is to determine at what point during stem cell differentiation do risks attributable to tumorigenic potential and hyperplasia become insignificant, if ever. Successful identification of the precise stage in the differentiation process of these hES cells, when the risk for tumor formation is no longer a safety concern, will depend on whether or not cellular differentiation proceeds in a unidirectional manner, or is reversible. Due to inherent inefficiencies in the biological process of differentiation, it is unlikely that phenotypic maturation induced in cultures of undifferentiated stem cells will be complete. Accordingly, it is essential that analytical techniques are developed that permit careful evaluation of cell preparations derived from stem cells, in order to determine the number of residual undifferentiated cells, and partially differentiated intermediates, that are present. Judicious preclinical studies of appropriate duration that involve implantation of undifferentiated or partially-differentiated stem cells into immunocompromised animals should be carried out prior to initiating clinical trials, in order to address the issue of unregulated growth potential of a candidate stem cell-based therapy and its relationship to the process of cell differentiation. The propensity for more pluripotent, less differentiated stem cells to form histologically "benign" structures in anatomically sensitive locations, such as the enclosed space of the spinal cord or the neuropil of the central nervous system, presents a significant safety concern that must be evaluated.

As previously stated, proof-of-concept studies are important for characterizing the dose-response relationship of the intended stem cell-based therapy as it pertains to both activity and safety outcomes. Unlike small molecule pharmaceuticals, or recombinant therapeutic proteins, standard kinetic profiles for product metabolism and excretion are not applicable to stem cell-based products. Moreover, once delivered the stem cell-based product is not readily retrievable and thus, dose-dependent adverse effects may be difficult to control. It is important to bear in mind that the fate of cells postimplant, i.e., survival, migration, structural and functional integration, and phenotypic plasticity could potentially impact the safety and/or activity profile for a stem cell-based product. Thus, employing a range of doses of the stem cell-based product in all animal studies is highly recommended, to establish both a safety and activity profile. Similarly, developing strategies for effectively controlling cell dose levels after implantation of stem cell-based products could serve to improve the safety profile for this category of products. Potential solutions include ongoing efforts to introduce genetic switches into preparations of stem cells capable of regulating cellular proliferation that may be turned on or off using noninvasive, extrinsic means after the cells have been implanted.

SUMMARY

Stem cells are among the most complex biological therapeutic entities proposed for clinical use to date. In this chapter, a regulatory review approach used by the FDA to assess the safety of novel stem cell-based products has been described. Primary responsibility for evaluating the safety of investigational new treatments comprised of stem cells and their derivatives is assigned to the FDAs Center for Biologics Evaluation and Research, Office of Cellular, Tissue and Gene Therapies. Outfitted with a comprehensive approach developed for the regulation of tissue and cellular products, staffed by scientific reviewers with expertise in cell and developmental biology, and endowed with a wealth of experience in the evaluation of submissions involving administration of stem cell preparations to patients, the FDA is well-positioned to successfully meet its obligation for assuring that investigational human clinical trials are conducted

in as safe a manner as is reasonably achievable. With respect to experimental treatments involving stem cell-based products, safety assurance is fundamentally about demonstrating control over every facet of the cell therapy preparation process, beginning with the acquisition of source donor materials, comprehensive, detailed characterization of the derivative stem cell product, and ultimately implementation of adequate and appropriate preclinical testing. In counseling academic clinical investigators and biotechnology sponsors about particulars concerning individual stem cell development programs, the FDA recognizes the need to maintain flexibility in its recommendations, taking into consideration a variety of impact factors including the intended target patient population, the severity of the specific disease under study, along with the potential risks and benefits attributable to the investigational stem cell-based product. The agency regularly updates and re-evaluates recommendations applicable to production and testing of stem cell products, based on the accumulation of scientific and clinical experience along with feedback obtained from a variety of valued sources. In order to be credible and effective, any approach taken for assessing the safety of stem cell-based products must consider the dynamic complexity of their biology. Operating within the jurisdiction of its mandated legal authority, the FDA strives to meet its obligation of ensuring the safety of nascent biological products consisting of or derived from stem cells, by relying on an approach that encourages iterative and collaborative interactions with the innovators, and which is updated based on the best available science.

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KEY WORDS

cGMPs (current Good Manufacturing Practices) The most up-to-date scientifically sound methods, practices or principles that are implemented and documented during product development and production to ensure consistent manufacture of safe, pure and potent products.

Identity The use of physical or chemical characteristics of the product, inspection by macroscopic or microscopic methods, specific culture tests, of *in vivo* or *in vitro* tests sufficient to identify a product and distinguish it from any other product being processed in the same laboratory. [21 CFR 610.14]

Manufacture All steps involved in the propagation or processing and preparation of therapeutic products including, but not limited to filling, testing, labeling, packaging, and storage as performed by the manufacturer [21 CFR 600.3(t)].

Potency The specific ability or capacity of a therapeutic product to elicit an expected result, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended [21 CFR 600.3(s)].

Purity Relative freedom from extraneous matter in the finished product introduced during the manufacturing process, whether or not harmful to the recipient or deleterious to the product [21 CFR 600.3(r)].

Stability Ability of a product to retain its sterility, identity, purity, and potency over time when stored and shipped in designated packaging and under specified conditions.

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Chapter 69

It's Not About Curiosity, It's About Cures: People Help Drive Progress

Mary Tyler Moore with S. Robert Levine

CHOOSING LIFE

> Ah, but a man's reach should exceed his grasp. Or what's a heaven for?
>
> Robert Browning, "Adrea del Sarto"

Many of you know that I have had type 1 (juvenile) diabetes for nearly 40 years. As a consequence, I struggle every day, like millions of others, to do what happens naturally for people who do not have diabetes: achieve a balance between what I eat, the energy I expend, and the amount of insulin I inject. Although to most, metabolic balance is as automatic as breathing, to people with type 1 diabetes, like me, it requires constant vigilance, constant factoring and adjusting, frequent finger sticks to check blood sugars, and multiple daily insulin injections just to stay alive. Even with the greatest of care, and closest of personal scrutiny, I find that often I am unable to achieve good balance—my sugars are dangerously low or frighteningly high. Yes, dangerous and frightening—because, frankly, serious lows can lead to seizures, coma, and death; and highs, over time, result in life-limiting and life-shortening complications like blindness, amputation, kidney failure, heart disease, and stroke. Diabetes is an all too personal time bomb which can go off today, tomorrow, next year, or 10 years from now—a time bomb that affects millions, and must be defused.

This reality is made all too clear by the recent sudden death of a young friend, Danielle Alberti. Danielle was 31. She was an aspiring artist. Although rapidly losing her vision because of diabetic retinopathy, Danielle stuck to her dream of being a painter, and was pursuing her career when she, like too many young adults with type 1 diabetes, developed kidney failure. People with diabetes-related kidney failure do not do well on dialysis, so a kidney transplant was her only real option. With her doctor's guidance, she and her mother decided to return home together to Australia, where her chances for a transplant were greater. However, Danielle did not survive the flight. She died at 30 000 feet, seeking comfort in her mother's arms—her last words, "Mum, hold me."

Most of us share at least a piece of this experience—our loved ones, in times of pain or need, reaching out, looking to us for comfort, for a way to stop their suffering. At that moment, we would do anything in our power to change their reality, to take their pain from them. When given the choice or the power to effect change, we would choose to protect the lives of those we love. This is the quest we join, together, when we contemplate the promise of stem cell research, debate its proper methods, and work toward making our hopes a reality—curing disease and disability through stem cell-derived therapies. We choose the idea of a better life, and reach beyond our grasp to achieve it.

SIZE OF THE PROMISE

> It is not unrealistic to say that (stem cell) research has the potential to revolutionize the practice of medicine and improve the quality and length of life.
>
> Dr. Harold Varmus, Nobel laureate and former Director of the US National Institutes of Health (NIH)

Judgment does not only require choosing between the good and the bad. It often challenges us to balance more than one good, or choose between the bad and the worse. Good judgment, therefore, demands that we make efforts to understand the relative impact of our choices, to come to an understanding of the greater good.

However, measuring the promise of stem cell research, for me, does not start with the recitation of the literally hundreds of millions of people who could benefit from the insights gained and therapies derived. Instead, it starts with understanding its potential for you and me, our parents and children, our friends and families, neighbors, and co-workers.

For people with type 1 diabetes, we look first to stem cell research as a means to help us replace the insulin-producing cells of the pancreas that are destroyed by our

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disease. However, it also may provide insights into the genetic basis of diabetes, including the differences between type 1 diabetes, which is an autoimmune disease like lupus and multiple sclerosis, and the more common, obesity-related, type 2 diabetes. It may also provide solutions to the devastating complications of diabetes: blindness, kidney failure, amputation, and cardiovascular disease. For people with Parkinson's disease, stem cell research holds the promise of replacing destroyed specialized brain cells, and thereby freeing patients from the prison of disease-induced rigidity. For spinal injury patients, it offers the potential for regeneration of neural tissue, which would reconnect the pathways of sensation and motor control, and allow them to walk again, or talk again, or hug their child again. For people with heart failure, stem cell research may mean sleeping through the night without struggling for breath, dancing with a spouse, working in the garden, or sustaining one's job and independence. For the person with macular degeneration it might offer sight. Stem cell research offers hope for people with a great diversity of illnesses, for people of all ages and genders, and all backgrounds. It offers hope for each of us, and that hope is not measured by numbers. It is ultimately personal.



FIGURE 69-1 Juvenile Diabetes Research Foundation (JDRF) founding moms, Lee Ducat and Carol Lurie.

PERSONAL PROMISES FUEL PROGRESS

> Never, never, never, give up.
>
> Sir Winston Churchill

I have had the privilege of serving as the International Chairman of the Juvenile Diabetes Research Foundation (JDRF) (www.jdrf.org) since the mid-1980s (Figure 69-1). JDRF was founded in 1970 by the parents of children with type 1 diabetes. They were not satisfied with the only option offered to their children by health professionals—a lifetime of insulin injections just to stay alive, and the constant fear of the life-stealing complications they would face in the future. Insulin was not a cure, they knew it, and they wanted someone to do something about it.

So they challenged the established professional associations to do something, to think anew, and to invest in more research (especially type 1 diabetes research). They were brushed aside, but not bowed. They may have been "just moms and dads," but they had a purpose that was highly personal. Each of them had promised their child, their loved one, that they would do all they could to find a cure. They intended to keep that promise, and they have. In the years since its founding, JDRF has grown to be the largest charitable contributor to diabetes research in the world, providing over $1.4 billion in direct funding since 1975, including over $600 million in the last five years alone. However, the impact of this "people-driven" effort to find a cure has been far greater than just the dollars raised for research. JDRF families (supported by an extraordinary professional staff, many of whom have a direct connection to diabetes) have been key leaders in public advocacy that has resulted in the following:

1. The Diabetes Research and Education Act (mid-1970s) that established The National Institute of Diabetes Digestive and Kidney Diseases at the National Institutes of Health (NIH), and called for substantive increases in funding of diabetes research. At the time the NIH was only investing $18 million per year in diabetes research. It now spends more than $1.0 billion per year.
2. Congressional earmarks for research into the genetics of diabetes, and diabetes-related kidney disease (1980s).
3. Lifting the ban on fetal tissue research in 1993, which President Clinton did "for Sam," a young man with type 1 diabetes.
4. Doubling the NIH budget in five years. Between 1998 and 2003 the budget increased from approximately $13 billion per year to more than $26 billion per year.
5. Establishment of the congressionally-mandated Diabetes Research Working Group that reports to Congress periodically on progress in diabetes research, on research needs and opportunities for the future, and on adequacy of funding (1998).



FIGURE 69-2 Mary Tyler Moore (with Michael J. Fox) testifying before Congress in support of federal funding of stem cell research. (Photo courtesy of Larry Lettera/Camera 1.)

6. The Special Diabetes Initiative (1998–2011) that will provide by fiscal year 2011, more than $1.5 billion in supplemental funding for special initiatives in type 1 diabetes research (on top of usual NIH appropriations), and an equal dollar amount (more than $1.5 billion) to fund special initiatives in diabetes care and education for Native Americans with diabetes.

JDRF was also a founder of the Coalition for the Advancement of Medical Research (CAMR), a diverse coalition of health-related organizations committed to sustaining federal funding of stem cell research. Working through CAMR and independently, JDRF volunteers and staff played a crucial role in 2001 in convincing the George W. Bush Administration and congressional leadership not to support a total ban on embryonic stem cell research in the US (Figure 69-2), and more recently leading public advocacy in building bi-partisan support for passage of H.R. 810—the Castle-DeGette Stem Cell Research Enhancement Act of 2005. JDRFers were also lead proponents of California's $3 billion stem-cell research/regenerative medicine initiative, as well as other state-based stem-cell research programs.

The experience of the JDRF, along with the HIV and AIDS community, the women's health movement, the Parkinson's Action Network, and other grass roots organizations, proves that in their quest to find a cure for their children and loved ones for whatever pains them, in their personal wars against disease, moms and dads, partners and spouses, and people who care will never give up. In fact, people personally affected by illness are the natural and necessary leaders of any global cure movement. They understand the urgency and are uniquely willing do anything required to ensure that their loved ones are freed of the burden of disease as soon as possible. For them, "failure is not an option," because their very survival is at stake.

HOPE VERSUS HYPE

> I am not discouraged, because every wrong attempt discarded is another step forward.
>
> Thomas A. Edison

What we know about any area of health or science is as much the "wisdom" accumulated through countless errors as it is the outcome of our research "successes." Furthermore, progress is often gained as much through the accidental collision of an unexpected finding and a willing mind as it is through the careful and detailed application of all that is "known."

What, then, drives the hope versus hype discussions regarding stem cell research? Do we know enough to project possibilities? Yes. Do we know enough to make assertions of a particular outcome by a certain date? No. Should this diminish our commitment to pushing the field forward to prove its potential? Certainly not. Too much is at stake for us to delay, or to apply unreasonable constraints, because we are worried we might be wrong or might be overestimating the potential. What if we are underestimating it?

Just as with any endeavor at the frontier of new worlds, there is risk in taking the next steps. However, we cannot shrink from these risks. Rather, we must—with proper deliberation and due consideration of the risks—chart our

course, prepare ourselves for dealing with the unexpected, and move forward.

GIVING LIFE

> If you save one life, you save the world.
>
> The Talmud

I understand that embryonic stem cell research raises concerns among people of good will, each trying to do what is right based on their very personal religious and moral beliefs. I have not shied from that personal soul searching, nor has JDRF in its policy making, nor should anyone. I have found comfort in my heartfelt view that human stem cell research is truly life affirming. It is a direct outcome of a young family making a choice, without coercion or compensation, to donate a fertilized egg not used for *in vitro* fertilization, for research. An egg that otherwise would have been discarded or frozen forever. Because of the great potential of stem cell research, donating unused fertilized eggs is much like the life-giving choice a mother, whose child has died tragically in an automobile accident, makes when donating her child's organs to save another mother's child. It is the true pinnacle of charity to give so totally, so freely of oneself, to give life to another. Public support for stem cell research is an extension of this affirmation of life, and is the best way to ensure that it is undertaken with the highest ethical standards.

FRIENDS IN NEED AND DEED

LISA, 12 YEARS OLD

El Paso, Texas

Lisa has had type 1 diabetes since she was 5 years old. "It makes me sad to watch my parents cry when they tell people about my diabetes," she says. "I feel really sad when I think about the stuff that can happen if a cure isn't found soon. I don't want to go blind and never again see the faces of the people I love or the really pretty sunsets in El Paso. I don't want my legs amputated because I love to dance. I don't want to die early and have heart and kidney disease. I want a cure before any of these terrible complications can happen."

NICHOLAS, 4 YEARS OLD

Boca Raton, Florida

Nicholas was diagnosed with type 1 diabetes when he was 20 months old. Most nights, his mother, Rose Marie, must intervene in some way to keep Nicholas' blood sugar in the normal range. "As I hold him in the middle of the night, trying to coax him awake so I can feed him, I realize just how delicately his life hangs in the balance," Rose Marie says. "I rededicate myself to doing everything within my power to find a cure for this disease, which robs my son, and millions of others like him, of a healthy, carefree childhood, and which carries the constant threat of danger—like a thief in the night lurking to strike."

KYLIE, 12 YEARS OLD

Ogden, Utah

Kylie was diagnosed at age 8. "Over Christmas vacation last year, I had a friend in my fifth grade class who died," she says. "He had diabetes just like me. During the middle of the night he went into insulin shock and never woke up." Kylie fears the same thing could happen to her. "I would love to have a guarantee that the rest of my life will be long and normal, but I know that there is a lot of work still to be done before they can find a cure."

BRENNAN, 6 YEARS OLD, AND TANNER, 8 YEARS OLD

Round Rock, Texas

Brennan, 6, was diagnosed with type 1 diabetes at the age of 2, just seven months before his older brother Tanner was diagnosed. "Each day is filled with adversity and challenge," mom Amy says. "Will the disease win today? Will Brennan succumb to too-low blood sugar levels and lose consciousness? Will Tanner have his first seizure?"

COREY, 11 YEARS OLD

Secaucus, New Jersey

Corey has had type 1 diabetes since the age of 5. Sitting at home and complaining about diabetes, he says, won't change things, or get your voice heard, or get us closer to a cure. Since the age of 7, he has spoken at schools and fundraisers to tell his story of what it is like to be very young and have to live with diabetes. "I've found that while millions have this disease, many people don't have a clue about what it really means to live with it each day," he says. He talks about the adjustments and fears that diabetes has brought to him and his family, adding that "it has not made me afraid to do what I want to do in life, but I known it *will* make things harder." At the moment of his sixth grade graduation, Corey says, "I want to leave a legacy to help … other children as they strive to live a normal life and hope they'll never forget that we will find a cure."

EMILY, 15 YEARS OLD

Houston, Texas

Diagnosed with type 1 diabetes nine years ago, Emily says each day is a battle. At first the finger pricks and shots were the most difficult thing that Emily had to handle, but now she has bigger fears. "Now the hardest part is facing the reality



FIGURE 69-3 Mary Tyler Moore at the Juvenile Diabetes Research Foundation's (JDRF's) Children's Congress of 2001. Joined on stage by Larry King, Tony Bennett, John McDonough, George Nethercutt, R-WA, Alan Silvestri, and child delegates from 50 states. (Photo courtesy of Larry Lettera/Camera 1.)

that there is no cure, and that my life could be determined by this awful disease." Emily is a straight A student, and plays on her school's field hockey team. Her goal is to one day be an orthopedic surgeon—and to help find a cure for diabetes. "I and many others will not be able to rest until we find a cure," she says. "A cure for diabetes will not just happen; it must be pursued, researched, and fought for."

Each of these courageous children, supported by their families, were delegates to the JDRF's 2003 Children's Congress, where they met their elected leaders and advocated for a cure for themselves and all children (and adults) like them with type 1 diabetes (Figure 69-3).

Excerpted, with permission, from the JDRF 2003 Children's Congress Yearbook.

PEOPLE DRIVE PROGRESS

> I know of no safe depository of the ultimate powers of the society but the people themselves; and if we think them not enlightened enough to exercise their control with a wholesome discretion, the remedy is not to take it from them, but to inform their discretion by education.
>
> Thomas Jefferson

In science, politics, even religion, the public often cedes decisions, important decisions, to an "expertocracy" or to dogma. Perhaps this is out of respect, humility, fear, or unfamiliarity—even out of a presumption of incapacity. It is, however, a well-informed public that is most capable of making decisions in its own interest and that, uniquely, has the power to effect change. It is, therefore, a true test of leadership to cede discretion back to people and bring comfort to public decision-making through careful and objective expert counsel, access to a broad base of information, and support for taking specific actions to achieve goals.

This approach has defined the JDRF success. From its inception, the JDRF has been unique in the way that it conducts its review of research grants, and how it decides what research is funded. Scientific experts (peer reviewers) are joined by people personally affected by diabetes (lay reviewers) in the discussion of all proposed research. Scientific merit is established in these peer/lay collaborative review sessions. Then the lay reviewers meet separately, to discuss which of the meritorious grants are most responsive to the needs of people with diabetes—that is, which are most likely to have the greatest impact on finding a cure or reducing the burden of diabetes and its complications. It is the people personally affected by diabetes who make the final decisions of what to fund and what not to fund. The decisions of this lay review group are made in the context of cure goals and research priorities established by the JDRF Board, which is itself predominantly made up of people personally affected by diabetes. These goals and priorities were derived by the Board via a process of knowledge mapping, which was conducted by JDRF volunteers, staff, and expert advisors. Knowledge mapping identifies the current state of science along the many potential paths to a cure, where obstacles remain, and where there

is the greatest opportunity for JDRF investment to make a difference.

The experience at JDRF infers that decisions regarding embryonic stem cell research are best made in the open, with the full engagement of the public, and with particular attention to presentation of the broadest breadth of available information and opinion. We can be confident that the powers of society (in overseeing the conduct of science) can be safely ceded to the discretion of a well-informed populace.

BETTER HEALTH FOR ALL

> William Bradford, speaking in 1630 of the founding of the Plymouth Bay Colony, said that all great and honorable actions are accompanied with great difficulties, and both must be enterprised and overcome with answerable courage. If … our progress teaches us anything, it is that man, in his quest for knowledge and progress, is determined and cannot be deterred.
>
> President John F. Kennedy, speech at Rice University September 12, 1962

People like me who struggle daily with disease or disability, and the people who love us, recognize the difficulties of scientific advancement, accept the challenges, and every day answer with courage—if not for our own sake, for our children and our children's children. We are motivated not by curiosity, but by our dedication to finding cures. New therapies derived from embryonic stem cell research, conducted with public support by scientists from all areas of the globe, and made available to all who might benefit, are part of our broader vision of better health for all.

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